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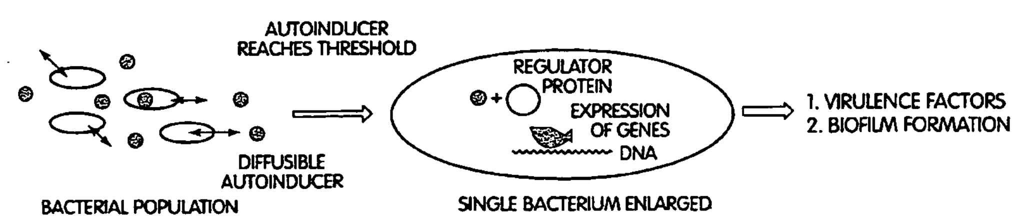


(57) Abstract: The invention provides methods for identifying a modulator of quorum sensing signaling in bacteria, and for identifying a quorum sensing controlled gene in bacteria. In addition, the invention provides quorum sensing controlled genetic loci in (*Pseudomonas aeruginosa*. Novel indicator strains and vectors for engineering the strains for use in the method of the invention are also provided.

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# QUORUM SENSING SIGNALING IN BACTERIA

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## Background of the Invention

Bacteria communicate with each other to coordinate expression of specific genes in a cell density dependent fashion. This "bacterial signaling" is a phenomenon called quorum sensing and response. Quorum sensing enables a bacterial species to sense its own number and regulate gene expression according to population density. In other words, quorum sensing is cell density-dependent regulation of genes that involves a freely diffusible molecule synthesized by the cell called an autoinducer (Fuqua, W.C. *et al.* (1996) *Annu. Rev. Microbiol.* 50:727-751; Salmond, G.P.C. *et al.* (1995) *Mol. Microbiol.* 16:615-624; Sitnikov, D.M. *et al.* (1995) *Mol. Microbiol.* 17:801-812). Autoinducers are described , *e.g.*, in U.S. Patents 5,591,872 and 5,593,827.

The paradigm system for quorum sensing is the *lux* system of the luminescent marine bacterium, *Vibrio fischeri*. *V. fischeri* exists at low cell densities in sea water and also at very high cell densities within the light organs of various marine organisms. such as the squid *Euprymna scolopes* (Pesci, E.C. *et al.*(1997) *Trends in Microbiol.* 5(4):132-135; Pesci, E.C. *et al.* (1997) *J. Bacteriol.* 179:3127-3132; Ruby, E.G. (1996) *Ann. Rev. Microbiol.* 50:591-624). At high cell densities, the *V. fischeri* genes encoding the enzymes required for light production are expressed. These genes are part of the *lux ICDABEG* operon and are regulated by the gene products of *luxI and luxR* (Baldwin, T.O. *et al.* (1989) *J. of Biolum. and Chemilum.* 4:326-341; Eberhard, A., *et al.* (1991) *Arch. of Microbiol.* 155:294-297; Gray, K.M. *et al.* (1992) *J. Bacteriol.* 174:4384-4390).

LuxI is an autoinducer synthase that catalyzes the formation of the *V. fischeri* autoinducer (VAI), *N*-(3oxohexanoyl) homoserine lactone (Eberhard, A., *et al.* (1991) *Arch. of Microbiol.* 155:294-297; Seed, P.C. *et al.* (1995) *J. Bacteriol.* 177:654-659). The autoinducer freely diffuses across the cell membrane and at high cell densities, reaches a critical concentration (Kaplan, H.B. *et al.* (1985) *J. Bacteriol.* 163:1210-

1214). At this critical concentration, VAI interacts with LuxR, a DNA-binding transcriptional regulator. The LuxR-VAI complex then binds to an upstream sequence of the *lux* operon called the "lux box", and activates transcription (Devine, J.H. *et al.* (1989) *PNAS* 86: 5688-5692; Hanzelka, B.A. *et al.* (1995) *J Bacteriol.* 177:815-817; Stevens, A.M. *et al.* (1994) *PNAS* 91:12619-12623). Since one of the genes of the operon is *luxI*, an autoregulatory loop is formed.

Many gram-negative bacteria have been shown to possess one or more quorum sensing systems (Fuqua, W.C. *et al.* (1996) *Annu. Rev. Microbiol.* 50:727-751; Salmond, G.P.C. *et al.* (1995) *Mol. Microbiol.* 16:615-624). These systems regulate a variety of physiological processes, including the activation of virulence genes and the formation of biofilms. The systems typically have acylated homoserine lactone ring autoinducers, in which the homoserine lactone ring is conserved. The acyl side chain, however, can vary in length and degree of substitution. In addition, it has been recently demonstrated that quorum sensing is involved in biofilm formation (Davies, D. G. *et al.* (1998) *Science.* 280(5361):295-8).

Biofilms are defined as an association of microorganisms, single or multiple species, that grow attached to a surface and produce a slime layer that provides a protective environment (Costerton, J. W. (1995) *J Ind Microbiol.* 15(3):137-40, Costerton, J. W. *et al.* (1995) *Annu Rev Microbiol.* 49:711-45). Typically, biofilms produce large amounts of extracellular polysaccharides, responsible for the slimy appearance, and are characterized by an increased resistance to antibiotics (1000- to 1500-fold less susceptible). Several mechanisms are proposed to explain this biofilm resistance to antimicrobial agents (Costerton, J. W. *et al.* (1999) *Science.* 284(5418):1318-22). One idea is that the extracellular matrix in which the bacterial cells are embedded provides a barrier toward penetration by the biocides. A further possibility is that a majority of the cells in a biofilm are in a slow-growing, nutrient-starved state, and therefore not as susceptible to the effects of anti-microbial agents. A third mechanism of resistance could be that the cells in a biofilm adopt a distinct and protected biofilm phenotype, *e.g.*, by elevated expression of drug-efflux pumps.

In most natural settings, bacteria grow predominantly in biofilms. Biofilms of *P. aeruginosa* have been isolated from medical implants, such as indwelling urethral, venous or peritoneal catheters (Stickler, D. J. *et al.* (1998) *Appl Environ Microbiol.*

64(9):3486-90). Chronic *P. aeruginosa* infections in cystic fibrosis lungs are considered to be biofilms (Costerton, J. W. *et al.* (1999) *Science.* 284(5418):1318-22).

In industrial settings, the formation of biofilms is often referred to as 'biofouling'. Biological fouling of surfaces is common and leads to material degradation, product contamination, mechanical blockage, and impedance of heat transfer in water-processing systems. Biofilms are also the primary cause of biological contamination of drinking water distribution systems, due to growth on filtration devices.

As noted earlier, many gram-negative bacteria have been shown to possess one or more quorum sensing systems that regulate a variety of physiological processes, including the activation of virulence genes and biofilm formation. One such gram negative bacterium is *Pseudomonas aeruginosa.*

*P. aeruginosa* is a soil and water bacterium that can infect animal hosts. Normally, the host defense system is adequate to prevent infection. However, in immunocompromised individuals (such as burn patients, patients with cystic fibrosis, or patients undergoing immunosuppressive therapy), *P. aeruginosa* is an opportunistic pathogen, and infection with *P. aeruginosa* can be fatal (Govan, J. R. *et al.* (1996) *Microbiol Rev.* 60(3):539-74; Van Delden, C. *et al.* (1998) *Emerg Infect Dis.* 4(4):551-60).

For example, Cystic fibrosis (CF), the most common inherited lethal disorder in Caucasian populations (~1 out of 2,500 life births), is characterized by bacterial colonization and chronic infections of the lungs. The most prominent bacterium in these infections is *P. aeruginosa*—by their mid-twenties, over 80% of people with CF have *P. aeruginosa* in their lungs (Govan, J. R. *et al.* (1996) *Microbiol Rev.* 60(3):539-74). Although these infections can be controlled for many years by antibiotics, ultimately they "progress to mucoidy," meaning that the *P. aeruginosa* forms a biofilm that is resistant to antibiotic treatment. At this point the prognosis is poor. The median survival age for people with CF is the late 20s, with *P. aeruginosa* being the leading cause of death (Govan, J. R. *et al.* (1996) *Microbiol Rev.* 60(3):539-74). According to the Cystic Fibrosis Foundation, treatment of CF cost more than $900 million in 1995 (Foundation, CF http://www.cff.org/homeline199701.htm).

*P. aeruginosa* is also one of several opportunistic pathogens that infect people with AIDS, and is the main cause of bacteremia (bacterial infection of the blood) and pneumonitis in these patients (Rolston, K. V. *et al.* (1990) *Cancer Detect Prev.* 14(3):377-81; Witt, D. J. *et al.* (1987) *Am J Med.* 82(5):900-6). A recent study of 1635 AIDS patients admitted to a French hospital between 1991-1995 documented 41 cases of severe *P. aeruginosa* infection (Meynard, J. L. *et al.* (1999) *J Infect.* 38(3):176-81). Seventeen of these had bacteremia, which was lethal in 8 cases. Similar, numbers were obtained in a smaller study in a New York hospital, where the mortality rate for AIDS patients admitted with *P. aeruginosa* bacteremia was about 50% (Mendelson, M. H. *et al.* 1994. *Clin Infect Dis.* 18(6):886-95).

In addition, about two million Americans suffer serious burns each year, and 10,000-12,000 die from their injuries. The leading cause of death is infection (Lee, J. J. *et al.* (1990) *J Burn Care Rehabil.* 11(6):575-80). *P. aeruginosa* bacteremia occurs in 10 % of seriously burned patients, with a mortality rate of 80 % (Mayhall, C. G. (1993) p. 614-664, Prevention and control of nosocomial infections. Williams & Wilkins, Baltimore; McManus, A. T *et al.* (1985) *Eur J Clin Microbiol.* 4(2):219-23).

Such infections are often acquired in hospitals ("nosocomial infections") when susceptible patients come into contact with other patients, hospital staff, or equipment. In 1995 there were approximately 2 million incidents of nosocomial infections in the U.S., resulting in 88,000 deaths and an estimated cost of $ 4.5 billion (Weinstein, R. A. (1998) *Emerg Infect Dis.* 4(3):416-20). Of the AIDS patients mentioned above who died of *P. aeruginosa* bacteremia, more than half acquired these infections in hospitals (Meynard, J. L. *et al.* (1999) *J Infect.* 38(3):176-81).

Nosocomial infections are especially common in patients in intensive care units as these people often have weakened immune systems and are frequently on ventilators and/or catheters. Catheter-associated urinary tract infections are the most common nosocomial infection (Richards, M. J. *et al.* (1999) *Crit Care Med.* 27(5):887-92) (31 % of the total), and *P. aeruginosa* is highly associated with biofilm growth and catheter obstruction. While the catheter is in place, these infections are difficult to eliminate (Stickler, D. J. *et al.* (1998) *Appl Environ Microbiol.* 64(9):3486-90). The second most frequent nosocomial infection is pneumonia, with *P. aeruginosa* the cause of infection in 21 % of the reported cases (Richards, M. J. *et al.* (1999) *Crit Care Med.* 27(5):887-

92). The annual costs for diagnosing and treating nosocomial pneumonia has been estimated at greater than $2 billion (Craven, D. E. *et al.* (1991) *Am J Med.* 91(3B):44S-53S).

Treatment of these so-called nosocomial infections is complicated by the fact that bacteria encountered in hospital settings are often resistant to many antibiotics. In June 1998, the National Nosocomial Infections Surveillance (NNIS) System reported increases in resistance of *P. aeruginosa* isolates from intensive care units of 89 % for quinolone resistance and 32 % for imipenem resistance compared to the years 1993-1997 (NNIS. http://www.cdc.gov/ncidod/hip/ NNIS/AR_Surv1198.htm). In fact, some strains of *P. aeruginosa* are resistant to over 100 antibiotics (Levy, S. (1998) *Scientific American.* March). There is a critical need to overcome the emergence of bacterial strains that are resistant to conventional antibiotics (Travis, J. (1994) *Science.* 264:360-362).

*P. aeruginosa* is also of great industrial concern (Bitton, G. (1994) Wastewater Microbiology. Wiley-Liss, New York, NY; Steelhammer, J. C. *et al.* (1995) *Indust. Water Treatm.*:49-55). The organism grows in an aggregated state, the biofilm, which causes problems in many water processing plants. Of particular public health concern are food processing and water purification plants. Problems include corroded pipes, loss of efficiency in heat exchangers and cooling towers, plugged water injection jets leading to increased hydraulic pressure, and biological contamination of drinking water distribution systems (Bitton, G. (1994) Wastewater Microbiology. Wiley-Liss, New York, NY, 9). The elimination of biofilms in industrial equipment has so far been the province of biocides. Biocides, in contrast to antibiotics, are antimicrobials that do not possess high specificity for bacteria, so they are often toxic to humans as well. Biocide sales in the US run at about $ 1 billion per year (Peaff, G. (1994) *Chem. Eng. News*:15-23).

A particularly ironic connection between industrial water contamination and public health issues is an outbreak of *P. aeruginosa* peritonitis that was traced back to contaminated poloxamer-iodine solution, a disinfectant used to treat the peritoneal catheters. *P. aeruginosa* is commonly found to contaminate distribution pipes and water filters used in plants that manufacture iodine solutions. Once the organism has matured into a biofilm, it becomes protected against the biocidal activity of the iodophor

solution. Hence, a common soil organism that is harmless to the healthy population, but causes mechanical problems in industrial settings, ultimately contaminated antibacterial solutions that were used to treat the very people most susceptible to infection.

Regulation of virulence genes by quorum sensing is well documented in *P. aeruginosa*. Recently, genes not directly involved in virulence including the stationary phase sigma factor *rpoS* and genes coding for components of the general secretory pathway (*xcp*) (Jamin, M. *et al.* (1991) *Biochem J.* 280(Pt 2):499-506) have been reported to be positively regulated by quorum sensing. Furthermore, the *las* quorum sensing system is required for maturation of *P. aeruginosa* biofilms (Chapon-Herve, V. *et al.* (1997) *Mol. Microbiol.* 24, 1169-1170; Davies, D. G.,*et al.* (1998) *Science* 280, 295-298). Thus it seems clear that quorum sensing represents a global gene regulation system in *P. aeruginosa*. However, the number and types of genes controlled by quorum sensing have not been identified or studied extensively.

## Summary of the Invention

In general, the invention pertains to the modulation of bacterial cell-to-cell signaling. The inhibition of quorum sensing signaling renders a bacterial population more susceptible to treatment, either directly through the host immune-response or in combination with traditional antibacterial agents and biocides. More particularly, the invention also pertains to a method for identifing modulators, *e.g.*, inhibitors of cell-to-cell signaling in bacteria, and in particular one particular human pathogen, *Pseudomonas aeruginosa*.

Thus, in one aspect, the invention is a method for identifying a modulator of quorum sensing signaling in bacteria. The method comprises:

providing a cell comprising a quorum sensing controlled gene, wherein the cell is responsive to a quorum sensing signal molecule such that a detectable signal is generated;

contacting said cell with a quorum sensing signal molecule in the presence and absence of a test compound;

and detecting a change in the detectable signal to thereby identify the test compound as a modulator of quorum sensing signaling in bacteria.

In one embodiment the cell comprises a reporter gene operatively linked to a regulatory sequence of a quorum sensing controlled gene, such that the quorum sensing signal molecule modulates the transcription of the reporter gene, thereby providing a detectable signal.

Another aspect of the invention is a method for identifying a modulator of a quorum sensing signaling in *Pseudomonas aeruginosa*. The method comprises:

providing a wild type strain of *Pseudomonas aeruginosa* which produces a quorum sensing signal molecule;

providing a mutant strain of *Pseudomonas aeruginosa* which comprises a reporter gene operatively linked to a regulatory sequence of a quorum sensing controlled gene, wherein the mutant strain is responsive to the quorum sensing signal molecule produced by the wild type strain, such that a detectable signal is generated;

contacting the mutant strain with the quorum sensing signal molecule and a test compound; and

detecting a change in the detectable signal to thereby identify the test compound as a modulator of quorum sensing signaling in *Pseudomonas aeruginosa.*

In one embodiment, the endogenous *lasI* and *rhlI* quorum sensing systems are inactivated in the mutant strain of *Pseudomonas aeruginosa*. In another embodiment the mutant strain of *Pseudomonas aeruginosa* comprises a promoterless reporter gene inserted at a genetic locus in the chromosome, wherein the genetic locus comprises a nucleotide sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35 and SEQ ID NO:36.

A further aspect of the invention is a mutant strain of *Pseudomonas aeruginosa* comprising a promoterless reporter gene inserted at a genetic locus in the chromosome, wherein the genetic locus comprises a nucleotide sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID

NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35 and SEQ ID NO:36.

In one embodiment, the endogenous *lasI* and *rhlI* quorum sensing systems are inactivated in the mutant strain of *Pseudomonas aeruginosa*. In another embodiment the mutant strain of *Pseudomonas aeruginosa* is responsive to a quorum sensing signal molecule such that a detectable signal is generated by the reporter gene. In yet another embodiment, the reporter gene is contained in a transposable element.

Yet another aspect of the invention is a method for identifying a modulator of quorum sensing signaling in *Pseudomonas aeruginosa*. The method comprises:

providing a wild type strain of *Pseudomonas aeruginosa* which produces a quorum sensing signal molecule;

providing a mutant strain of *Pseudomonas aeruginosa* which comprises a promoterless reporter gene inserted at a genetic locus in the chromosome of said *Pseudomonas aeruginosa,* wherein the genetic locus comprises a nucleotide sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35 and SEQ ID NO:36; and wherein the mutant strain is responsive to the quorum sensing signal molecule produced by the wild type strain, such that a detectable signal is generated by the reporter gene;

contacting the mutant strain with the quorum sensing signal molecule and a test compound; and

detecting a change in the detectable signal to thereby identify the test compound as a modulator of quorum sensing signaling in *Pseudomonas aeruginosa.*

Another aspect of the invention is an isolated nucleic acid molecule comprising a nucleotide sequence which comprises:

a regulatory sequence derived from the genome of *Pseudomonas aeruginosa*, wherein the regulatory sequence regulates a quorum sensing controlled genetic locus of the *Pseudomonas aeruginosa* chromosome, and wherein the genetic locus comprises a nucleotide sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35 and SEQ ID NO:36; and

a reporter gene operatively linked to the regulatory sequence.

A further aspect of the invention provides an isolated nucleic acid molecule comprising a quorum sensing controlled genetic locus derived from the genome of *Pseudomonas aeruginosa*, wherein the genetic locus comprises a nucleotide sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35 and SEQ ID NO:36, operatively linked to a reporter gene.

In one embodiment, the invention is an isolated nucleic acid molecule comprising a polynucleotide having at least 80% identity to a quorum sensing controlled genetic locus derived from the genome of *Pseudomonas aeruginosa*, wherein the genetic

locus comprises a nucleotide sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35 and SEQ ID NO:36, operatively linked to a reporter gene.

In another embodiment, the invention is an isolated nucleic acid molecule comprising a a polynucleotide that hybridizes under stringent conditions to a quorum sensing controlled genetic locus derived from the genome of *Pseudomonas aeruginosa*, wherein the genetic locus comprises a nucleotide sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35 and SEQ ID NO:36, operatively linked to a reporter gene.

In one embodiment, an isolated nucleic acid molecule of the invention comprises a reporter gene contained in a transposable element.

Accordingly, a further aspect of the invention pertains to a vector comprising an isolated nucleic acid molecule of the invention. In another aspect, the invention provides cells containing an isolated nucleic acid molecule of the invention.

An additional aspect of the invention is a method for identifying a modulator of quorum sensing signaling in bacteria. The method comprises:

providing a cell containing an isolated nucleic acid molecule of the invention, wherein the cell is responsive to a quorum sensing signal molecule such that a detectable signal is generated;

contacting said cell with a quorum sensing signal molecule in the presence and absence of a test compound;

and detecting a change in the detectable signal to therby identify the test compound as a modulator of quorum sensing signaling in bacteria.

Accordingly, in another aspect, the invention provides a compound identified by a method of the invention which modulates, *e.g.*, inhibits, quorum sensing signaling in *Pseudomonas aeruginosa.* In one embodiment, the compound inhibits quorum sensing signaling in *Pseudomonas aeruginosa* by inhibiting an enzyme involved in the synthesis of a quorum sensing signal molecule, by interfering with quorum sensing signal reception, or by scavenging the quorum sensing signal molecule.

The invention also pertains to a method for identifing quorum sensing controlled genes in a cell, and specifically in one particular human pathogen, *Pseudomonas aeruginosa.* Thus, in one aspect, the invention provides a method for identifying a quorum sensing controlled gene in a cell, the method comprising:

providing a cell which is responsive to a quorum sensing signal molecule such that expression of a quorum sensing controlled gene is modulated, and wherein modulation of the expression of said quorum sensing controlled gene generates a detectable signal;

contacting said cell with a quorum sensing signal molecule;

and detecting a change in the detectable signal to thereby identify a quorum sensing signaling controlled gene.

In one embodiment the cell comprises a reporter gene operatively linked to a quorum sensing controlled gene or a regulatory sequence of a quorum sensing controlled gene, such that modulation of the expression of the quorum sensing controlled gene modulates the transcription of the reporter gene, thereby providing a detectable signal. In another embodiment the reporter gene is contained in a transposable element. In yet another embodiment, the quorum sensing signal molecule is produced by a second cell, *e.g.*, a bacterial cell. In a further embodiment, the quorum sensing signal molecule is an autoinducer of said quorum sensing controlled gene, *e.g.*, a homoserine lactone, or an analog thereof.

## Brief Description f the Drawings

*Figure 1* depicts the paragdigm for quorum sensing signaling in the target bacterium, *Pseudomonas aeruginosa.*

*Figure 2* depicts patterns of β-galactosidase expression in representative qsc mutants and in a strain with a *lasB::lacZ* chromosomal fusion generated by site-specific mutation. Units of β-galactosidase are given as a function of culture density for cells grown without added signal molecules (○), with added $3OC_{12}$-HSL (●), with added $C_4$-HSL (■), or with both signals added (□).

*Figure 3* depicts the nucleic acid sequence of the quorum sensing controlled locus on the *P. aeruginosa* chromosome mapped in the *P. aeruginosa* mutant strain qsc102.

*Figure 4* depicts putative qsc operons. Open reading frames (ORFs) are indicated by the arrows. ORFs discovered in the qsc screen are indicated by their qsc number.

*Figure 5* depicts a growth curve of PAO1/pMW303G. Culture growth is monitored at 600 nm (closed circles) and β-galactosidase activity is measured with a chemiluminescent substrate analog in relative light units (RLU; open circles).

*Figure 6* is a map of the qsc insertions on the *P. aeruginosa* chromosome. Arrowheads indicate the direction of *lacZ* transcription. In addition to the qsc mutants, *lasR* and *lasI*, *rhlR*, and *lasB* are also mapped. The locations of las-boxes like elements are shown as black dots between the two DNA strands. The numbers indicate distance in megabases on the approximately 6 megabase chromosome.

*Figure 7* depicts putative *las*-type boxes in upstream DNA regions of qsc mutants. ORFs as described in Materials and Methods. Bases outlined in black represent residues conserved in all sequences and gray outlines are conserved in 8 of 10 sequences.

*Figure 8* depicts the principle of a bioassay for modulators of quorum sensing signaling. Strain PAO1 produces the signal 3-oxo-C12-HSL. Strain QSC102 responds by inducing *lacZ*.

*Figure 9* depicts the results of an assay performed using the test compound acetyl-butyrolactone, which is present in the wells at increasing concentration (mM, as indicated). There are two rows and two columns per concentration to show reproducibily of the assay.

*Figure 10A* depicts the structure of a mobilizable plasmid for generating an indicator strain. Filled boxes represent chromosomal DNA derived from the *P. aeruginosa* locus where *lacZ* is inserted in strain QSC102.

*Figure 10B* depicts induction of ß-galactosidase as PAQ1 reaches high density. Cell growth is monitored at 600 nm (closed circles) and expression of ß-galactosidase is measured in Miller units (open circles).

*Figure 11* depicts the reaction mechanism of the RhlI autoinducer synthase.

*Figure 12* depicts a continuous culture bioreactor.

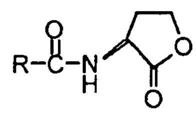
**<u>Detailed Description of the Invention</u>**

In gram-negative bacteria, such as *Pseudomonas aeruginosa*, quorum sensing involves two proteins, the autoinducer synthase - the I protein - and the transcriptional activator - the R protein. The synthase produces an acylated homoserine lactone (the "autoinducer"; see structure 1 below), which can diffuse into the surrounding environment (Fuqua, C. *et al.* (1998) *Curr Opin Microbiol.* <u>1</u>(2):183-189; Fuqua, *et al.* 1994. *J Bacteriol.* <u>176</u>(2):269-75). The autoinducer molecule is composed of an acyl chain in a peptide bond with the amino nitrogen of a homoserine lactone (HSL). For different quorum sensing systems, the side-chain may vary in length, degree of saturation, and oxidation state. As the density of bacteria increases, so does the concentration of this freely diffusible signal molecule. Once the concentration reaches a

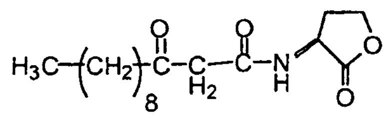
defined threshold, it binds to the R-protein, which then activates transcription of numerous genes. Of particular interest are genes involved in pathogenicity and in biofilm formation (see Figure 1).

*Pseudomonas aeruginosa* has two quorum sensing systems, *las* and *rhl*, named for their role in the expression of elastase, and the RhlI/RhlR proteins, which were first described for their role in rhamnolipid biosynthesis. (Hanzelka, B.A. *et al.* (1996) *J. Bacteriol.* 178:5291-5294; Baldwin, T.O. *et al.* (1989) *J. of Biolum. and Chemilum.* 4:326-341; Passador, L., *et al.* (1993) *Science* 260:1127-1130; Pearson, J.P *et al.* (1994) *PNAS* 91:197-201; Pesci, E.C. *et al.*(1997) *Trends in Microbiol.* 5(4):132-135; Pesci, E.C. *et al.* (1997) *J. Bacteriol.* 179:3127-3132). The two systems have distinct autoinducer synthases (*lasI* and *rhlI*), transcriptional regulators (*lasR* and *rhlR*), and autoinducers (*N*-(3-oxododecanoyl) homoserine lactone (HSL) and *N*-butyryl HSL) (Sitnikov. D.M. *et al.* (1995) *Mol. Microbiol.* 17:801-812). The *rhl* and *las* systems are involved in regulating the expression of a number of secreted virulence factors, biofilm development, and the stationary phase sigma factor (RpoS) (Davies, D.G. *et al.* (1998) *Science* 280:295-298; Latifi, A. *et al.* (1995) *Mol. Microbiol. Rev.* 17:333-344; Ochsner, U.A., *et al.* (1995) *PNAS*, 92:6424-6428; Pesci, E.C. *et al.*(1997) *Trends in Microbiol.* 5(4):132-135; Pesci, E.C. *et al.* (1997) *J. Bacteriol.* 179:3127-3132). Expression of the *rhl* system requires a functional *las* system, therefore the two systems in combination with RpoS constitute a regulatory cascade (Pesci. E.C. *et al.*(1997) *Trends in Microbiol.* 5(4):132-135; Pesci. E.C. *et al.* (1997) *J. Bacteriol.* 179:3127-3132, Seed *et al.* 1995).

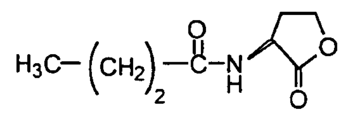
The signal in the Las system is 3-oxo-dodecanoyl-HSL (3-oxo-C12-HSL) **2**, while the signal used in the Rhl system is butanoyl-HSL (C4-HSL) **3**. It has been shown that 3-oxo-C12-HSL increases expression of RhlR, indicating a hierarchy of regulation systems (Pesci, E. C. *et al.* (1997) *Trends Microbiol.* 5(4):132-4). The Las signal 3-oxo-C12-HSL is synthesized by LasI along with a small amount of *N*-(3-oxooctanoyl) HSL and *N*-(3-oxohexanoyl) HSL, while RhlI makes primarily the signal C4-HSL and a small amount of *N*-hexanoyl (Pearson, J.P.*et al.* (1997) *J. Bacteriol.* 179:5756-5757; Winson, M.K. *et al.* (1995) *PNAS* 92:9427-9431).



**1**: acylated HSL



**2**: 3-oxo-dodecanoyl-HSL



**3**: butanoyl-HSL

Bacterial signaling triggers the expression of a number of virulence factors in *P. aeruginosa* including two elastases, an alkaline protease and exotoxin A (Pesci, E. C. *et al.* (1997) *Trends Microbiol.* 5(4):132-4; Pesci, E. C. *et al.* (1997) *J Bacteriol.* 179(10):3127-32) - proteins that allow the organism to attack host tissue. Bacterial signaling also controls the expression of the antioxidant pyocyanin, a compound that allows the bacteria to neutralize one important host defense, the generation of superoxide radicals (Britigan, *et al.* (1999) *Infect Immun.* 67(3):1207-12, Hassan, H. M. *et al.* (1979) *Arch Biochem Biophys.* 196(2):385-95, Hassan, H. M. *et al.* 1980. *J Bacteriol.* 141(1):156-63). It has been shown in a neonatal mouse model that a defined mutant of *P. aeruginosa* which lacks the signal receptor protein (LasR) was significantly less virulent than the wild type PAO1, as measured by the ability to cause acute pneumonia, bacteremia and death (Tang, H. B. *et al.* (1996) *Infect Immun.* 64(1):37-43).

The invention is based on the interruption of bacterial cell-to-cell singaling, *i.e.*, quorum sensing signaling in order to render a bacterial population more susceptible to treatment, either through the host immune-response or in combination with traditional antibacterial agents and biocides. Thus, the invention provides a bacterial indicator strain that allows for a high throughput screening assay for identifying compounds that modulate, *e.g.*, inhibit bacterial cell-to-cell signalling. The compounds so identified will provide novel anti-pathogenics and anti-fouling agents.

**Definitions**

Before further description of the invention, certain terms employed in the specification, examples and appended claims are, for convenience, collected here.

The term "analog" as in "homoserine lactone analog" is intended to encompass compounds that are chemically and/or electronically similar but have different atoms, such as isosteres and isologs. An analog includes a compound with a structure similar to that of another compound but differing from it in respect to certain components or structural makeup. The term analog is also intened to encompass stereoisomers.

The language "autoinducer compounds" is art-recognized and is intended to include molecules, *e.g.*, proteins which freely diffuse across cell membranes and which activate transcription of various factors which affect bacterial viability. Such compounds can affect virulence, and biofilm development. Autoinducer compounds can be acylated homoserine lactones. They can be other compounds similar to those listed in Table 1. Homoserine autoinducer compounds are produced *in vivo* by the interaction of a homoserine lactone substrate and an acylated acyl carrier protein in a reaction catalyzed by an autoinducer synthase molecule. In isolated form, autoinducer compounds can be obtained from naturally occurring proteins by purifying cellular extracts, or they can be chemically synthesized or recombinantly produced.
The language "autoinducer synthase molecule" is intended to include molecules, *e.g.* proteins, which catalyze or facilitate the synthesis of autoinducer compounds, *e.g.* in the quorum sensing system of bacteria. It is also intended to include active portions of the autoinducer synthase protein contained in the protein or in fragments or portions of the protein (*e.g.*, a biologically active fragment). The language "active portions" is intended to include the portion of the autoinducer synthase protein which contains the homoserine lactone binding site.

Table 1 contains a list of exemplary autoinducer synthase proteins of the quorum sensing systems of various gram-negative bacteria.

**Table 1.** Summary of *N*-acyl homoserine lactone based regulatory systems

| **Bacterial species** | **Signal molecules**[a] | **Regulatory Proteins**[b] | **Target function(s)** |
|---|---|---|---|
| *Vibrio fischeri* | N-3-(oxohexanoyl)-homoserine lactone (VAI-1) | LuxI/LuxR | *luxI*CDABEG. *lux*R luminescence |
| | N-(octanoyl)-L-homoserine lactone (VAI-2) | AinS/AinR[c] | *luxI*CDABEG. ? |
| *Vibrio harveyi* | *N*-*β*-(hydroxybutyryl)-homoserine lactone (HAI-1) | LuxM/LuxN-LuxO-LuxR[d] | *luxI*CDABEG. luminescence and polyhydroxybutyrate synthesis |
| | HAI-2 | Lux?/LuxPQ-LuxO-LuxR[d] | *lux*CDABEG |
| *Pseudomonas aeruginosa* | N-3-(oxododecanyoyl)-L-homoserine lactone (PAI-1) | LasI/LasR | *las*B. *las*A. *apr*A. *tox*A. virulence factors |
| | N-(butyryl)-L-homoserine lactone (PAI-2) | RhlI/RhlR | *rhl*AB. rhamnolipid synthesis. virulence factors |
| *Pseudomonas aeureofaciens* | (PRAI)[e] | PhzI/PhzR | *phz*. phenazine biosynthesis |
| *Agroacterium tumefaciens* | N-3-(oxooctanoyl)-L-homoserine lactone (AAI) | TraI/TraR-TraM | *tra* gens. *tra*R. Ti plasmid conjugal transfer |
| *Erwinia carotovora subsp. carotovora* SCRI193 | VAI-1[f] | ExpI/ExpR | *pel, pec, pep,* exoenzyme synthesis |
| *Erwinia carotovora subsp. carotovora* SCC3193 | VAI-1[f] | CarI/CarR | *cap,* carbapenem antibiotic synthesis |

| | | | |
|---|---|---|---|
| *Erwinia carotovora subsp. carotovora* 71 | VAI-1[f] | HslI/? | *pel, pec, pep,* exoenzyme synthesis |
| *Erwinia stewartii* | VAI-1[f] | EsaI/EsaR | *wts* genes. exopolysaccharide synthesis, virulence factors |
| *Rhizobium leguminosarum* | *N*-(3R-hydroxy-7-*cis*-tetradecanoyl-L-homoserine lactone. small bacteriocin. (RLAI) | ?/RhiR | *rhi*ABC. rhizosphere genes and stationary phase |
| *Enterobacter agglomerans* | VAI-1[f] | EagI/EagR | function unclear |
| *Yersenia enterocolitica* | VAI-1[f] | YenI/YenR | function unclear |
| *Serratia liquifaciens* | *N*-butanoyl-L-homoserine lacton (SAI-1) | SwrI/? | swarming motility |
| | *N*-hexanoyl-L-homoserine lacton (SAI-2) | SwrI/? | swarming motility |
| *Aeromonas hydrophila* | (AHAI)[e] | AhyI/AhyR | function unclear |
| *Escherichia coli/?*[g] | | ?/SdiA | *fts*QAZ. cell division |

Autoinducer synthase molecules can be obtained from naturally occurring sources, *e.g.*, by purifying cellular extracts, can be chemically synthesized or can be recombinantly produced. Recombinantly produced autoinducer synthase molecules can have the amino acid sequence of a a naturally occurring form of the autoinducer synthase protein. They can also have a similar amino acid sequence which includes mutations such as substitutions and deletions (including truncation) of a naturally occurring form of the protein. Autoinducer synthase molecules can also include molecules which are structurally similar to the structures of naturally occurring autoinducer synthase proteins, *e.g.*, biologically active variants.

TraI, LuxI, RhlI are the homoserine lactone autoinducer synthases of *Agrobacterium tumefaceins*, *Vibrio fischeri*, and *Pseudomonas aeruginosa*, respectively. The term "RhlI" is intended to include proteins which catalyze the synthesis of the homoserine lactone autoinducer of the RhlI quorum sensing system of *P. aeruginosa*, butyryl homoserine lactone.

The term "biofilm" is intended to include biological films that develop and persist at interfaces in aqueous environments. Biofilms are composed of microorganisms embedded in an organic gelatinous structure composed of one or more matrix polymers which are secreted by the resident microorganisms. The language "biofilm development" or "biofilm formation" is intended to include the formation, growth, and modification of the bacterial colonies contained with the biofilm structures as well as the synthesis and maintenance of the exopolysaccharide matrix of the biofilm structures.

The term "compound" as used herein (*e.g.*, as in "test compound," or "modulator compound") is intended to include both exogenously added test compounds and peptides endogenously expressed from a peptide library. Test compounds may be purchased, chemically synthesized or recombinantly produced. Test compounds can be obtained from a library of diverse compounds based on a desired activity, or alternatively they can be selected from a random screening procedure. In one embodiment, an indicator cell (*e.g.*, a cell which responds to quorum sensing signals by generating a detectable signal) also produces the test compound which is being screened. For instance, the indicator cell can produce, *e.g.*, a test polypeptide, a test nucleic acid and/or a test carbohydrate, which is screened for its ability to modulate quorum sensing signaling. In such embodiments, a culture of such reagent cells will collectively provide a library of potential modulator molecules and those members of the library which either stimulate or inhibit quorum sensing signaling can be selected and identified. In another embodiment, a test compound is produced by a second cell which is co-incubated with the indicator cell.

The terms "derived from" or "derivative", as used interchangeably herein, are intended to mean that a sequence is identical to or modified from another sequence, *e.g.*, a naturally occurring squence. Derivatives within the scope of the invention include polynucleotide derivatives. Polynucleotide or nucleic acid derivatives differ from the sequences described herein (*e.g.*, SEQ ID Nos.: 1-38) or known in nucleotide sequence. For example, a polynucleotide derivative may be characterized by one or more nucleotide substitutions, insertions, or deletions, as compared to a reference sequence. A nucleotide sequence comprising a quorum sensing controlled genetic locus that is derived from the genome of *P. aeruginosa*, *e.g.*, SEQ ID Nos.: 1-38, includes sequences

that have been modified by various changes such as insertions, deletions and substitutions, and which retain the property of being regulated in response to a quorum sensing signaling event. Such sequences may comprise a quorum sensing controlled regulatory element and/or a quorum sensing controlled gene. The nucleotide sequence of the *P. aeruginosa* genome is available at www.pseudomonas.com.

Polypeptide or protein derivatives include polypeptide or protein sequences that differ from the sequences described or known in amino acid sequence, or in ways that do not involve sequence, or both, and still preserve the activity of the polypeptide or protein. Derivatives in amino acid sequence are produced when one or more amino acids is substituted with a different natural amino acid, an amino acid derivative or non-native amino acid. In certain embodiments protein derivatives include naturally occurring polypeptides or proteins, or biologically active fragments thereof, whose sequences differ from the wild type sequence by one or more conservative amino acid substitutions, which typically have minimal influence on the secondary structure and hydrophobic nature of the protein or peptide. Derivatives may also have sequences which differ by one or more non-conservative amino acid substitutions, deletions or insertions which do not abolish the biological activity of the polypeptide or protein.

Conservative substitutions (substituents) typically include the substitution of one amino acid for another with similar characteristics (*e.g.*, charge, size, shape, and other biological properties) such as substitutions within the following groups: valine, glycine; glycine, alanine; valine, isoleucine; aspartic acid, glutamic acid; asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine. The non-polar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

In other embodiments, derivatives with amino acid substitutions which are less conservative may also result in desired derivatives, *e.g.*, by causing changes in charge, conformation and other biological properties. Such substitutions would include, for example, substitution of hydrophilic residue for a hydrophobic residue, substitution of a cysteine or proline for another residue, substitution of a residue having a small side

chain for a residue having a bulky side chain or substitution of a residue having a net positive charge for a residue having a net negative charge. When the result of a given substitution cannot be predicted with certainty, the derivatives may be readily assayed according to the methods disclosed herein to determine the presence or absence of the desired characteristics. The polypeptides and proteins of this invention may also be modified by various changes such as insertions, deletions and substitutions, either conservative or nonconservative where such changes might provide for certain advantages in their use.

As used herein, the term "genetic locus" includes a position on a chromosome, or within a genome, which is associated with a particular gene or genetic sequences having a particular characteristic. For example, in one embodiment, a quorum sensing controlled genetic locus includes nucleic acid sequences which comprise an open reading frame (ORF) of a quorum sensing controlled gene. In another embodiment, a quorum sensing controlled genetic locus includes nucleic acid sequences which comprise transcriptional regulatory sequences that are responsive to quorum sensing signaling (*e.g.*, a quorum sensing controlled regulatory element). Examples of quorum sensing controlled genetic loci of *P. aeruginosa* are described herein as SEQ ID NOs.:1-38.

The term "modulator", as in "modulator of quorum sensing signaling" is intended to encompass, in its various grammatical forms, induction and/or potentiation, as well as inhibition and/or downregulation of quorum sensing signaling and/or quorum sensing controlled gene expression. As used herein, the term "modulator of quorum sensing signaling" includes a compound or agent that is capable of modulating or regulating at least one quorum sensing controlled gene or quorum sensing controlled genetic locus, *e.g.*, a quorum sensing controlled genetic locus in *P. aeruginosa*, as described herein. A modulator of quorum sensing signaling may act to modulate either signal generation (*e.g.*, the synthesis of a quorum sensing signal molecule), signal reception (*e.g.*, the binding of a signal molecule to a receptor or target molecule), or signal transmission (*e.g.*, signal transduction via effector molecules to generate an appropriate biological response). In one embodiment, a method of the present invention encompasses the modulation of the transcription of an indicator gene in response to an autoinducer molecule. In another embodiment, a method of the present invention encompasses the

modulation of the transcription of an indicator gene, preferably an quorum sensing controlled indicator gene, by a test compound.

The term "operatively linked" or "operably linked" is intended to mean that molecules are functionally coupled to each other in that the change of activity or state of one molecule is affected by the activity or state of the other molecule. In one embodiment, nucleotide sequences are "operatively linked" when the regulatory sequence functionally relates to the DNA sequence encoding the polypeptide or protein of interest. For example, a nucleotide sequence comprising a transcriptional regulatory element(s) (*e.g.*, a promoter) is operably linked to a DNA sequence encoding the protein or polypeptide of interest if the promoter nucleotide sequence controls the transcription of the DNA sequence encoding the protein of interest. In addition, two nucleotide sequences are operatively linked if they are coordinately regulated and/or transcribed. Typically, two polypeptides that are operatively linked are covalently attached through peptide bonds.

The term "quorum sensing signaling" or "quorum sensing" is intended to include the generation of a cellular signal in response to cell density. In one embodiment, quorum sensing signaling mediates the coordinated expression of specific genes. A "quorum sensing controlled gene" is any gene, the expression of which is regulated in a cell density dependent fashion. In a preferred embodiment, the expression of a quorum sensing controlled gene is modulated by a quorum sensing signal molecule, *e.g.*, an autoinducer molecule (*e.g.*, a homoserine lactone molecule). The term "quorum sensing signal molecule" is intended to include a molecule that transduces a quorum sensing signal and mediates the cellular response to cell density. In a preferred embodiment the quorum sensing signal molecule is a freely diffusible autoinducer molecule, *e.g.*, a homoserine lactone molecule or analog thereof. In one embodiment, a quorum sensing controlled gene encodes a virulence factor. In another embodiment, a quorum sensing controlled gene encodes a protein or polypeptide that, either directly or indirectly, inhibits and/or antagonizes a bacterial host defense mechanism. In yet another embodiment, a quorum sensing controlled gene encodes a protein or polypeptide that regulates biofilm formation.

The term "regulatory sequences" is intended to include the DNA sequences that control the transcription of an adjacent gene. Gene regulatory sequences include, but are not limited to, promoter sequences that are found in the 5' region of a gene proximal to the transcription start site which bind RNA polymerase to initiate transcription. Gene regulatory sequences also include enhancer sequences which can function in either orientation and in any location with respect to a promoter, to modulate the utilization of a promoter, and other expression control elements (*e.g.*, polyadenylation signals). Such regulatory sequences are described, for example, in Goeddel (1990) *Methods Enzymol.* 185:3-7. Transcriptional control elements include, but are not limited to, promoters, enhancers, and repressor and activator binding sites. The gene regulatory sequences of the present invention contain binding sites for transcriptional regulatory proteins. In one embodiment, a regulatory sequence includes a sequence that mediates quorum sensing controlled gene expression, *e.g.*, a las box. In a preferred embodiment, gene regulatory sequences comprise sequences derived from the *Pseudomonas aeruginosa* genome which modulate quorum sensing controlled gene expression, *e.g.*, SEQ ID NOs.:38 and 39. In another preferred embodiment, gene regulatory sequences comprise sequences (*e.g.*, a genetic locus) derived from the *Pseudomonas aeruginosa* genome which modulate the expression of quorum sensing controlled genes, *e.g.*, SEQ ID NOs.:1-36.

The term "reporter gene" or "indicator gene" generically refers to an expressible (*e.g.*, able to be transcribed and (optionally) translated) DNA sequence which is expressed in response to the activity of a transcriptional regulatory protein. Indicator genes include unmodified endogenous genes of the host cell, modified endogenous genes, or a reporter gene of a heterologous construct, *e.g.*, as part of a reporter gene construct. In a preferred embodiment, the level of expression of an indicator gene produces a detectable signal.

Reporter gene constructs are prepared by operatively linking an indicator gene with at least one transcriptional regulatory element. If only one transcriptional regulatory element is included, it is advantageously a regulatable promoter. In a preferred embodiment at least one of the selected transcriptional regulatory elements is directly or indirectly regulated by quorum sensing signals, whereby quorum sensing controlled gene expression can be monitored via transcription and/or translation of the reporter genes.

Many reporter genes and transcriptional regulatory elements are known to those of skill in the art and others may be identified or synthesized by methods known to those of skill in the art. Reporter genes include any gene that expresses a detectable gene product, which may be RNA or protein. Preferred reporter genes are those that are readily detectable. In one embodiment, an indicator gene of the present invention is comprised in the nucleic acid molecule in the form of a fusion gene (*e.g.*, operatively linked) with a nucleotide sequence that includes regulatory sequences (*e.g.*, quorum sensing transcriptional regulatory elements, *e.g.*, a las box) derived from the *Pseudomonas aeruginosa* genome (*e.g.*, SEQ ID NOs:38 and 39). In another embodiment, an indicator gene of the present invention is operatively linked to quorum sensing transcriptional regulatory sequences that regulate a quorum sensing controlled genetic locus derived from the *Pseudomonas aeruginosa* genome, *e.g.*, a genetic locus comprising a nucleotide sequence set forth as SEQ ID NOs.: 1-36. In yet another embodiment, an indicator gene of the present invention is operatively linked to a nucleotide sequence comprising a quorum sensing controlled genetic locus derived from the *Pseudomonas aeruginosa* genome (*e.g.*, SEQ ID NOs.:1-39). In certain embodiments of the invention, an indicator gene (*e.g.*, a promoterless indicator gene) is contained in a transposable element.

The term "detecting a change in the detectable signal" is intended to include the detection of alterations in gene transcription of an indicator or reporter gene induced upon modulation of quorum sensing signaling. In certain embodiments, the reporter gene may provide a selection method such that cells in which the transcriptional regulatory protein activates transcription have a growth advantage. For example the reporter could enhance cell viability, relieve a cell nutritional requirement, and/or provide resistance to a drug. In other embodiments, the detection of an alteration in a signal produced by an indicator gene encompass assaying general, global changes to the cell such as changes in second messenger generation.

The amount of transcription from the reporter gene may be measured using any method known to those of skill in the art. For example, specific mRNA expression may be detected using Northern blots, or a specific protein product may be identified by a characteristic stain or an intrinsic activity. In preferred embodiments, the gene product of the reporter is detected by an intrinsic activity associated with that product. For

instance, the reporter gene may encode a gene product that, by enzymatic activity, gives rise to a detection signal based on color, fluorescence, or luminescence.

The amount of regulation of the indicator gene, *e.g.*, expression of a reporter gene, is then compared to the amount of expression in a control cell. For example, the amount of transcription of an indicator gene may be compared between a cell in the absence of a test modulator molecule and an identical cell in the presence of a test modulator molecule.

As used interchangeably herein, the terms "transposon" and "transposable element" are intended to include a piece of DNA that can insert into and cut itself out of, genomic DNA of a particular host species. Transposons include mobile genetic elements (MGEs) containing insertion sequences and additional genetic sequences unrelated to insertion functions (for example, sequences encoding a reporter gene). Insertion sequence elements include sequences that are between 0.7 and 1.8 kb in size with termini approximately 10 to 40 base pairs in length with perfect or nearly perfect repeats. As used herein, a transposable element is operatively linked to the nucleotide sequence into which it is inserted. Transposable elements are well known in the art, and are described for example, at www.bact.wisc.edu/MicrotextBook/BactGenetics.

The present invention discloses a method for identifying modulators of quorum sensing signaling in bacteria, e.g., *Pseudomonas aeruginosa*. As described herein, the method of the invention comprises providing a cell which comprises a quorum sensing controlled gene, wherein the cell is responsive to a quorum sensing signal molecule such that a detectable signal is generated. A cell which responds to a quorum sensing signal molecule by generating a detectable signal is referred to herein as an "indicator cell" or a "reporter cell". In a preferred embodiment of the invention, the cell is a *P. aeruginosa* bacterial cell. In another preferred embodiment, the cell is from a mutant strain of *P. aeruginosa* which comprises a reporter gene operatively linked to a regulatory sequence of a quorum sensing controlled gene, wherein said mutant strain is responsive to a quorum sensing signal molecule, such that a detectable signal is generated. In yet another preferred embodiment, the cell is a mutant strain of *P. aeruginosa* which comprises a promoterless reporter gene inserted in the chromosome at a quorum sensing controlled genetic locus, *e.g.*, a genetic locus comprising a nucleotide sequence set forth as SEQ ID NOs.:1-38, wherein said mutant strain is responsive to a quorum sensing

signal molecule such that a detectable signal is generated by the reporter gene. In a preferred embodiment, the reporter gene is contained in a transposable element. In a further preferred embodiment, the cell is from a strain of *P. aeruginosa* in which *lasI* and *rhlI* are inactivated, such that the cell does not express the *lasI* and *rhlI* autoinducer synthases which are involved in the generation of quorum sensing signal molecules. A compound is identified as a modulator of quorum sensing signaling in bacteria by contacting the cell with a quorum sensing signal molecule in the presence and absence of a test compound and detecting a change in the detectable signal.

Quorum sensing signal molecules that are useful in the methods of the present invention include autoinducer compounds such as homoserine lactones, and analogs thereof (see Table 1). In certain embodiments, the quorum sensing signal molecule is either 3-oxo-C12-homoserine lactone or C4-HSL. In one embodiment, the cell does not express the quorum sensing signal molecule. For example, the cell may comprise a mutant strain of *Pseudomonas aeruginosa* wherein *lasI* and *rhlI* are inactivated. Therefore, the cell is contacted with an exogenous quorum sensing signal molecule, *e.g.*, a recombinant or synthetic molecule. In another embodiment, the quorum sensing signal molecule is produced by a second cell (*e.g.*, a prokaryotic or eukaryotic cell), which is co-incubated with the indicator cell. For example, an indicator cell which does not express a quorum sensing signal molecule can be co-incubated with a wild type strain of *Pseudomonas aeruginosa* which produces a quorum sensing signal molecule. Alternatively, the indicator strain which does not express a quorum sensing signal molecule is co-incubated with a second cell which has been transformed, or otherwise altered, such that it is able to express a quorum sensing signal moleucle. In yet another embodiment, the quorum sensing signal molecule is expressed by the indicator strain.

Similarly, the test compound can be exogenously added to an indicator strain, produced by a second cell which is co-incubated with the indicator strain, or expressed by the indicator strain. Exemplary compounds which can be screened for activity include, but are not limited to, peptides, nucleic acids, carbohydrates, small organic molecules, and natural product extract libraries.

The test compounds of the present invention can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; spatially addressable parallel solid phase or solution phase libraries;

synthetic library methods requiring deconvolution; the 'one-bead one-compound' library method; and synthetic library methods using affinity chromatography selection. The biological library approach is limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam, K.S. (1997) *Anticancer Drug Des.* 12:45).

Examples of methods for the synthesis of molecular libraries can be found in the art, for example, in: DeWitt *et al.* (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90:6909; Erb *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:11422; Zuckermann *et al.* (1994). *J. Med. Chem.* 37:2678; Cho *et al.* (1993) *Science* 261:1303; Carrell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2059; Carell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2061; and Gallop *et al.* (1994) *J. Med. Chem.* 37:1233.

Libraries of compounds may be presented in solution (*e.g.*, Houghten (1992) *Biotechniques* 13:412-421), or on beads (Lam (1991) *Nature* 354:82-84), chips (Fodor (1993) *Nature* 364:555-556), bacteria (Ladner USP 5,223,409), spores (Ladner USP '409), plasmids (Cull *et al.* (1992) *Proc Natl Acad Sci USA* 89:1865-1869) or on phage (Scott and Smith (1990) *Science* 249:386-390); (Devlin (1990) *Science* 249:404-406); (Cwirla *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87:6378-6382); (Felici (1991) *J. Mol. Biol.* 222:301-310); (Ladner *supra.*).

In certain embodiments of the instant invention, the compounds tested are in the form of peptides from a peptide library. The peptide library may take the form of a cell culture, in which essentially each cell expresses one, and usually only one, peptide of the library. While the diversity of the library is maximized if each cell produces a peptide of a different sequence, it is usually prudent to construct the library so there is some redundancy. Depending on size, the combinatorial peptides of the library can be expressed as is, or can be incorporated into larger fusion proteins. The fusion protein can provide, for example, stability against degradation or denaturation. In an exemplary embodiment of a library for intracellular expression, *e.g.*, for use in conjunction with intracellular target receptors, the polypeptide library is expressed as thioredoxin fusion proteins (see, for example, U.S. Patents 5,270,181 and 5,292,646; and PCT publication WO94/ 02502). The combinatorial peptide can be attached on the terminus of the thioredoxin protein, or, for short peptide libraries, inserted into the so-called active loop.

In one embodiment of the instant invention the cell further comprises a means for generating the detectable signal. For example, the cell may comprise a reporter gene, the transcription of which is regulated by a quorum sensing signal molecule. In a preferred embodiment, the reporter gene is operatively linked to a regulatory sequence of a quorum sensing controlled gene, *e.g.* a nucleotide sequence comprising at least one quorum sensing controlled regulatory element, *e.g.*, a las box. In another embodiment, the reporter gene is operatively linked to a quorum sensing controlled genetic locus, *e.g.*, a quorum sensing controlled gene, such that transcription of the indicator gene is responsive to quorum sensing signals. For example, in a preferred embodiment, a promoterless reporter gene is inserted into a quorum sensing controlled genetic locus derived from the genome of *P. aeruginosa*. Such quorum sensing controlled genetic loci, as described herein, include the loci in the *P. aeruginosa* genome which comprise the nucleotide sequences set forth as SEQ ID NOs.: 1-38. In another preferred embodiment, the promoterless reporter gene is contained in a transposable element that is inserted into a quorum sensing controlled genetic locus in the *P. aeruginosa* genome.

Examples of reporter genes include, but are not limited to, CAT (chloramphenicol acetyl transferase) (Alton and Vapnek (1979), Nature 282: 864-869), and other enzyme detection systems, such as beta-galactosidase (*lacZ*), firefly luciferase (deWet *et al.* (1987), Mol. Cell. Biol. 7:725-737); bacterial luciferase (Engebrecht and Silverman (1984), PNAS 1: 4154-4158; Baldwin *et al.* (1984), Biochemistry 23: 3663-3667); alkaline phosphatase (Toh *et al.* (1989) Eur. J. Biochem. 182: 231-238, Hall *et al.* (1983) J. Mol. Appl. Gen. 2: 101), human placental secreted alkaline phosphatase (Cullen and Malim (1992) Methods in Enzymol. 216:362-368), and horseradish peroxidase. In one preferred embodiment, the indicator gene is lacZ. In another preferred embodiment, the indicator gene is green fluorescent protein (U.S. patent 5,491,084; WO96/23898) or a variant thereof. A preferred variant is GFPmut2. Other reporter genes include *ADE1, ADE2, ADE3, ADE4, ADE5, ADE7, ADE8, ASP3, ARG1, ARG3, ARG4, ARG5, ARG6, ARG8, ARO2, ARO7, BAR1, CAT, CHO1, CYS3, GAL1, GAL7, GAL10, HIS1, HIS3, HIS4, HIS5, HOM3, HOM6, ILV1, ILV2, ILV5, INO1, INO2, INO4, LEU1, LEU2, LEU4, LYS2, MAL, MEL, MET2, MET3, MET4, MET8, MET9, MET14, MET16, MET19, OLE1, PHO5, PRO1, PRO3, THR1, THR4, TRP1, TRP2, TRP3, TRP4, TRP5, URA1, URA2, URA3, URA4, URA5* and *URA10*.

In accordance with the methods of the invention, compounds which modulate quorum sensing singaling can be selected and identified. The ability of compounds to modulate quorum sensing signaling can be detected by up or down-regulation of the detection signal provided by the indicator gene. Any difference, *e.g.*, a statistically significant difference, in the amount of transcription indicates that the test compound has in some manner altered the activity of quorum sensing signaling.

A modulator of quorum sensing signaling may act by inhibiting an enzyme involved in the synthesis of a quorum sensing signal molecule, by inhibiting reception of the quorum sensing signal molecule by the cell, or by scavenging the quorum sensing signal molecule. The term "scavenging" is meant to include the sequestration, chemical modification, or inactivation of a quorum sensing signal molecule such that it is no longer able to regulate quorum sensing gene control. After identifying certain test compounds as potential modulators of quorum sensing signaling, the practitioner of the subject assay will continue to test the efficacy and specificity of the selected compounds both *in vitro* and *in vivo*, *e.g.*, in an assay for bacterial viability and/or pathogenecity.

In another aspect, the present invention discloses a method for identifying a quorum sensing controlled gene in bacteria, *e.g.*, *Pseudomonas aeruginosa*. The method comprises providing a cell which is responsive to a quorum sensing signal molecule such that expression of a quorum sensing controlled gene is modulated, and wherein modulation of the expression of the quorum sensing controlled gene generates a detectable signal. The cell is contacted with a quorum sensing signal molecule and a change in the signal is detected to thereby identify a quorum sensing signaling controlled gene.

In one embodiment, the cell further comprises a means for generating the detectable signal, *e.g.*, a reporter gene. For example, the cell may comprise a promoterless reporter gene that is operatively linked to a quorum sensing controlled genetic locus such that modulation of the expression of the quorum sensing controlled locus concurrently modulates transcription of the reporter gene. The position of the quorum sensing controlled genetic locus is then mapped based on the position of the reporter gene.

In a preferred embodiment of the invention, the cell is a *P. aeruginosa* bacterial cell. In another preferred embodiment, the cell is a mutant strain of *P. aeruginosa* which comprises a promoterless reporter gene inserted in the chromosome at a quorum sensing controlled genetic locus, *e.g.*, a genetic locus comprising a nucleotide sequence set forth as SEQ ID NOs.:1-39, wherein said mutant strain is responsive to a quorum sensing signal molecule such that a detectable signal is generated by the reporter gene. In a preferred embodiment, the reporter gene is contained in a transposable element. In a further preferred embodiment, the cell is from a strain of *P. aeruginosa* in which *lasI* and *rhlI* are inactivated, such that the cell does not express the *lasI* and *rhlI* autoinducer synthases which are involved in the generation of quorum sensing signal molecules.

It is also to be understood that genomic sequences from a mutant bacterial strain (*e.g.*, *P. aeruginosa*) in which a promoterless reporter gene (*e.g.*, a reporter gene contained in a transposable element) has been inserted at a quorum sensing controlled locus, can be assayed in a heterologous cell that is responsive to a quorum sensing signal molecule such that quorum sensing signal transduction occurs. For example, the genomic DNA of a strain of *P. aeruginosa* subjected to transposon mutagenesis, as described herein, can be engineered into a library, and transferred to another cell capable of quorum sensing signaling (*e.g.*, a different species of gram negative bacteria), and assayed to identify a quorum sensing controlled gene.

In one embodiment, the cell is contacted with an exogenous quorum sensing signal molecule, *e.g.*, a recombinant or synthetic molecule, as described herein. In another embodiment, the quorum sensing signal molecule is produced by a second cell (*e.g.*, a prokaryotic or eukaryotic cell), which is co-incubated with the indicator cell. For example, an indicator cell which does not express a quorum sensing signal molecule can be co-incubated with a wild type strain of *Pseudomonas aeruginosa* which produces a quorum sensing signal molecule. Alternatively, the indicator strain which does not express a quorum sensing signal molecule is co-incubated with a second cell which has been transformed, or otherwise altered, such that it is able to express a quorum sensing signal moleucle. In yet another embodiment, the quorum sensing signal molecule is expressed by the indicator strain.

Another aspect of the invention provides a mutant strain of *Pseudomonas aeruginosa* comprising a promoterless reporter gene inserted in a chromsome at a genetic locus comprising a nucleotide sequence set forth as SEQ ID NOs:1-36, *e.g.*, a quorum sensing controlled genetic locus. In one embodiment the reporter gene is contained in a transposable element. In another embodiment, the reporter gene is lacZ or GFP, or a variant thereof, *e.g.*, GFPmut2. In yet another embodiment, *lasI* and *rhlI* are inactivated in the mutant strain of *P. aeruginosa.* The above-described cells are useful in the methods of the instant invention, as the cells are responsive to a quorum sensing signal molecule such that a detectable signal is generated by the reporter gene. These cells are also useful for studying the function of polypeptides encoded by the quorum sensing controlled loci comprising the nucleotide sequences set forth as SEQ ID NOs.:1-36.

Yet another aspect of the invention provides isolated nucleic acid molecules comprising a nucleotide sequence comprising a quorum sensing controlled genetic locus derived from the genome of *Pseudomonas aeruginosa* operatively linked to a reporter gene. In one embodiment, a reproter gene is operatively linked to a regulatory sequence derived from the genome of *P. aeruginosa*, wherein the regulatory sequence regulates a quorum sensing controlled genetic locus comprising a nucleotide sequence set forth as SEQ ID NO:1-36. In a preferred embodiment such regulatory sequences comprise at least one binding site for a quorum sensing controlled transcriptional regulatory factor (*e.g.*, a transcriptional activator or repressor molecule) such that transcription of the reporter gene is responsive to a quorum sensing singal molecule and/or a modulator of quorum sensing signaling. In another embodiment, a reporter gene is operatively linked to a quorum sensing controlled genetic locus derived from the genome of *P. aeruginosa*, wherein the genetic locus comprises a nucleotide sequence set forth as SEQ ID NO:1-36. In yet another embodiment, a reporter gene is operatively linked to a nucleotide sequence which has at least 80%, and more preferably at least 85%, 90% or 95% identity to quorum sensing controlled genetic locus derived from the genome of *P. aeruginosa*, wherein the genetic locus comprises a nucleotide sequence set forth as SEQ ID NO:1-36. In a further embodiment, a reporter gene is operatively linked to a nucleotide sequence which hybridizes under stringent conditions to quorum sensing

controlled genetic locus derived from the genome of *P. aeruginosa*, wherein the genetic locus comprises a nucleotide sequence set forth as SEQ ID NO:1-36.

The term "isolated nucleic acid molecule" includes nucleic acid molecules which are separated from other nucleic acid molecules which are present in the natural source of the nucleic acid. For example, with regard to genomic DNA, the term "isolated" includes nucleic acid molecules which are separated from the chromosome with which the genomic DNA is naturally associated. Preferably, an "isolated" nucleic acid is free of sequences which naturally flank the nucleic acid (*i.e.*, sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, the isolated nucleic acid molecule can contain less than about 5 kb, 4kb, 3kb, 2kb, 1 kb, 0.5 kb or 0.1 kb of nucleotide sequences which naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived. Moreover, an "isolated" nucleic acid molecule, such as a cDNA molecule, can be substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized. As used interchangeably herein, the terms "nucleic acid molecule" and "polynucleotide" are intended to include DNA molecules (*e.g.*, cDNA or genomic DNA) and RNA molecules (*e.g.*, mRNA) and analogs of the DNA or RNA generated using nucleotide analogs. The nucleic acid molecule can be single-stranded or double-stranded, but preferably is double-stranded DNA. The term"DNA" refers to deoxyribonucleic acid whether single- or double-stranded. As used herein, the terms "gene" and "recombinant gene" refer to nucleic acid molecules which include an open reading frame encoding a protein, preferably a quorum sensing controlled protein, and can further include non-coding regulatory sequences, and introns.

The present invention includes polynucleotides capable of hybridizing under stringent conditions, prefereably highly stringent conditions, to the polynucleotides described herein (*e.g.*, a quorum sensing controlled genetic locus, *e.g.*, SEQ ID NOs.:1-36). As used herein, the term "hybridizes under stringent conditions" is intended to describe conditions for hybridization and washing under which nucleotide sequences that are significantly identical or homologous to each other remain hybridized to each other. Preferably, the conditions are such that sequences at least about 70%, more

preferably at least about 80%, even more preferably at least about 85% or 90% identical to each other remain hybridized to each other. Such stringent conditions are known to those skilled in the art and can be found in *Current Protocols in Molecular Biology*, Ausubel *et al.*, eds., John Wiley & Sons, Inc. (1995), sections 2, 4, and 6. Additional stringent conditions can be found in *Molecular Cloning: A Laboratory Manual*, Sambrook *et al.*, Cold Spring Harbor Press, Cold Spring Harbor, NY (1989), chapters 7, 9, and 11. A preferred, non-limiting example of stringent hybridization conditions includes hybridization in 4X sodium chloride/sodium citrate (SSC), at about 65-70°C (or alternatively hybridization in 4X SSC plus 50% formamide at about 42-50°C) followed by one or more washes in 1X SSC, at about 65-70°C. A preferred, non-limiting example of highly stringent hybridization conditions includes hybridization in 1X SSC, at about 65-70°C (or alternatively hybridization in 1X SSC plus 50% formamide at about 42-50°C) followed by one or more washes in 0.3X SSC, at about 65-70°C. A preferred, non-limiting example of reduced stringency hybridization conditions includes hybridization in 4X SSC, at about 50-60°C (or alternatively hybridization in 6X SSC plus 50% formamide at about 40-45°C) followed by one or more washes in 2X SSC, at about 50-60°C. Ranges intermediate to the above-recited values, *e.g.*, at 65-70°C or at 42-50°C are also intended to be encompassed by the present invention. SSPE (1xSSPE is 0.15M NaCl, 10mM $NaH_2PO_4$, and 1.25mM EDTA, pH 7.4) can be substituted for SSC (1X SSC is 0.15M NaCl and 15mM sodium citrate) in the hybridization and wash buffers; washes are performed for 15 minutes each after hybridization is complete. The hybridization temperature for hybrids anticipated to be less than 50 base pairs in length should be 5-10°C less than the melting temperature ($T_m$) of the hybrid, where $T_m$ is determined according to the following equations. For hybrids less than 18 base pairs in length, $T_m(°C) = 2(\# \text{ of } A + T \text{ bases}) + 4(\# \text{ of } G + C \text{ bases})$. For hybrids between 18 and 49 base pairs in length, $T_m(°C) = 81.5 + 16.6(\log_{10}[Na^+]) + 0.41(\%G+C) - (600/N)$, where N is the number of bases in the hybrid, and $[Na^+]$ is the concentration of sodium ions in the hybridization buffer ($[Na^+]$ for 1X SSC = 0.165 M). It will also be recognized by the skilled practitioner that additional reagents may be added to hybridization and/or wash buffers to decrease non-specific hybridization of nucleic acid molecules to membranes, for example, nitrocellulose or nylon membranes, including but not limited to blocking agents (*e.g.*, BSA or salmon or herring sperm carrier DNA),

detergents (*e.g.*, SDS), chelating agents (*e.g.*, EDTA), Ficoll, PVP and the like. When using nylon membranes, in particular, an additional preferred, non-limiting example of stringent hybridization conditions is hybridization in 0.25-0.5M $NaH_2PO_4$, 7% SDS at about 65°C, followed by one or more washes at 0.02M $NaH_2PO_4$, 1% SDS at 65°C (see *e.g.*, Church and Gilbert (1984) *Proc. Natl. Acad. Sci. USA* 81:1991-1995), or alternatively 0.2X SSC, 1% SDS.

The invention further encompasses nucleic acid molecules that differ from the quorum sensing controlled genetic loci described herein, *e.g.*, the nucleotide sequences shown in SEQ ID NO:1-36. Accordingly, the invention also includes variants, *e.g.*, allelic variants, of the disclosed polynucleotides or proteins; that is naturally occuring and non-naturally occuring alternative forms of the isolated polynucleotide which may also encode proteins which are identical, homologous or related to that encoded by the polynucleotides of the invention.

Nucleic acid variants can be naturally occurring, such as allelic variants (same locus), homologues (different locus), and orthologues (different organism) or can be non naturally occurring. Non-naturally occurring variants can be made by mutagenesis techniques, including those applied to polynucleotides, cells, or organisms. The variants can contain nucleotide substitutions, deletions, inversions and insertions. Variation can occur in either or both the coding and non-coding regions. The variations can produce both conservative and non-conservative amino acid substitutions (as compared in the encoded product). Allelic variants result, for example, from DNA sequence polymorphisms within a population (*e.g.*, a bacterial population) that lead to changes in the nucleic acid sequences of quorum sensing controlled genetic loci.

To determine the percent identity of two amino acid sequences or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (*e.g.*, gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). In a preferred embodiment, the length of a reference sequence aligned for comparison purposes is at least 30%, preferably at least 40%, more preferably at least 50%, even more preferably at least 60%, and even more preferably at least 70%, 80%, 90% or 95% of the length of the reference sequence. The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are

then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position (as used herein amino acid or nucleic acid "identity" is equivalent to amino acid or nucleic acid "homology"). The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. In a preferred embodiment, the percent identity between two amino acid sequences is determined using the Needleman and Wunsch (*J. Mol. Biol.* (48):444-453 (1970)) algorithm which has been incorporated into the GAP program in the GCG software package (available at http://www.gcg.com), using either a Blossom 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. In yet another preferred embodiment, the percent identity between two nucleotide sequences is determined using the GAP program in the GCG software package (available at http://www.gcg.com), using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. In another embodiment, the percent identity between two amino acid or nucleotide sequences is determined using the algorithm of E. Meyers and W. Miller (*Comput. Appl. Biosci.*, 4:11-17 (1988) which has been incorporated into the ALIGN program (version 2.0) (available at http://vega.igh.cnrs.fr/bin/align-guess.cgi), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4.

The nucleic acid and protein sequences of the present invention can further be used as a "query sequence" to perform a search against public databases to, for example, identify other family members or related sequences. Such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul, *et al.* (1990) *J. Mol. Biol.* 215:403-10. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to protein molecules of the invention. To obtain gapped

alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.*, (1997) *Nucleic Acids Res.* 25(17):3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (*e.g.*, XBLAST and NBLAST) can be used. See http://www.ncbi.nlm.nih.gov. Additionally, the "Clustal" method (Higgins and Sharp, Gene, 73:237-44, 1988) and "Megalign" program (Clewley and Arnold, *Methods Mol. Biol*, 70:119-29, 1997) can be used to align sequences and determine similarity, identity, or homology.

Accordingly, the present invention also discloses recombinant vector constructs and recombinant host cells transformed with said constructs.

The term "vector" or "recombinant vector" is intended to include any plasmid, phage DNA, or other DNA sequence which is able to replicate autonomously in a host cell. As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. A vector may be characterized by one or a small number of restriction endonuclease sites at which such DNA sequences may be cut in a determinable fashion without the loss of an essential biological function of the vector, and into which a DNA fragment may be spliced in order to bring about its replication and cloning. A vector may further contain a marker suitable for use in the identification of cells transformed with the vector. Recombinant vectors may be generated to enhance the expression of a gene which has been cloned into it. after transformation into a host. The cloned gene is usually placed under the control of (*i.e.*, operably linked to) certain control sequences or regulatory sequences, which may be either constitutive or inducible.

One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments can be ligated. Another type of vector is a viral vector. wherein additional DNA segments can be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e.g.*, bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "expression vectors". Expression systems for both

prokaryotic and eukaryotic cells are described in, for example, chapters 16 and 17 of Sambrook, J. *et al. Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

In the present specification, "plasmid" and "vector" can be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (*e.g.*, replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions. Protocols for producing recombinant retroviruses and for infecting cells *in vitro* or *in vivo* with such viruses can be found in Current Protocols in Molecular Biology, Ausubel, F.M. et al. (eds.) Greene Publishing Associates, (1989), Sections 9.10-9.14 and other standard laboratory manuals. Examples of suitable retroviruses include pLJ, pZIP, pWE and pEM which are well known to those skilled in the art. Examples of suitable packaging virus lines include ψCrip, ψCre, ψ2 and ψAm. The genome of adenovirus can be manipulated such that it encodes and expresses a transcriptional regulatory protein but is inactivated in terms of its ability to replicate in a normal lytic viral life cycle. See for example Berkner et al. (1988) *BioTechniques* 6:616; Rosenfeld et al. (1991) *Science* 252:431-434; and Rosenfeld et al. (1992) *Cell* 68:143-155. Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (*e.g.*, Ad2, Ad3, Ad7 etc.) are well known to those skilled in the art. Alternatively, an adeno-associated virus vector such as that described in Tratschin et al. ((1985) *Mol. Cell. Biol.* 5:3251-3260) can be used.

In general, it may be desirable that an expression vector be capable of replication in the host cell. Heterologous DNA may be integrated into the host genome, and thereafter is replicated as a part of the chromosomal DNA, or it may be DNA which replicates autonomously, as in the case of a plasmid. In the latter case, the vector will include an origin of replication which is functional in the host. In the case of an integrating vector, the vector may include sequences which facilitate integration, *e.g.*, sequences homologous to host sequences, or encoding integrases.

Appropriate cloning and expression vectors for use with bacterial, fungal, yeast, and mammalian cellular hosts are known in the art, and are described in, for example, Powels *et al.* (*Cloning Vectors: A Laboratory Manual.* Elsevier, New York, 1985).

Mammalian expression vectors may comprise non-transcribed elements such as an origin of replication, a suitable promoter and enhancer linked to the gene to be expressed, and other 5' or 3' flanking nontranscribed sequences, and 5' or 3' nontranslated sequences, such as necessary ribosome binding sites, a poly-adenylation site, splice donor and acceptor sites, and transcriptional termination sequences.

The vectors of the subject invention may be transformed into an appropriate cellular host for use in the methods of the invention.

As used interchangeably herein, a "cell" or a "host cell" includes any cultivatable cell that can be modified by the introduction of heterologous DNA. As used herein, "heterologous DNA", a "heterologous gene" or "heterologous polynucleotide sequence" is defined in relation to the cell or organism harboring such a nucleic acid or gene. A heterologous DNA sequence includes a sequence that is not naturally found in the host cell or organism, *e.g.*, a sequence which is native to a cell type or species of organism other than the host cell or organism. Heterologous DNA also includes mutated endogenous genetic seqeunces, for example, as such sequences are not naturally found in the host cell or organism. Preferably, a host cell is one in which a quorum sensing signal molecule, *e.g*, an autoinducer molecule, initiates a quorum sensing signaling response which includes the regulation of target quorum sensing controlled genetic sequences . The choice of an appropriate host cell will also be influenced by the choice of detection signal. For example, reporter constructs, as described herein, can provide a selectable or screenable trait upon activation or inhibition of gene transcription in response to a quorum sensing signaling event; in order to achieve optimal selection or screening, the host cell phenotype will be considered.

A host cell of the present invention includes prokaryotic cells and eukaryotic cells. Prokaryotes include gram negative or gram positive organisms, for example, *E. Coli* or *Bacilli.* Suitable prokaryotic host cells for transformation include, for example, *E. coli*. *Bacillus subtilis*, *Salmonella typhimurium*, and various other species within the genera *Pseudomonas*, *Streptomyces*, and *Staphylococcus*. In a preferred embodiment, a host cell of the invention is a mutant strain of *P. aeruginosa* in which *lasI* and *rhlI* are inactivated.

Eukaryotic cells include, but are not limited to, yeast cells, plant cells, fungal cells, insect cells (*e.g.*, baculovirus), mammalian cells, and cells of parasitic organisms, *e.g.*, trypanosomes. Mammalian host cell culture systems include established cell lines such as COS cells, L cells, 3T3 cells, Chinese hamster ovary (CHO) cells, embryonic stem cells, and HeLa cells. Other suitable host cells are known to those skilled in the art.

DNA can be introduced into prokaryotic or eukaryotic cells via conventional transformation or transfection techniques. As used herein, the terms "transformation" and "transfection" are intended to refer to a variety of art-recognized techniques for introducing foreign nucleic acid (*e.g.*, DNA) into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation. Suitable methods for transforming or transfecting host cells can be found in Sambrook, *et al.* (*Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989), and other laboratory manuals.

Host cells comprising an isolated nucleic acid molecule of the invention (*e.g.*, a quorum sensing controlled genetic locus operatively linked to a reporter gene) can be used in the methods of the instant invention to identify a modulator of quorum sensing signaling in bacteria.

## Exemplification

The invention is further illustrated by the following examples which should not be construed as limiting.

## EXAMPLE 1 IDENTIFICATION OF QUORUM SENSING GENES OF *P. AERUGINOSA*

### Materials and Methods

**Bacterial Strains, Plasmids, and Media.** The bacterial strains and plasmids used in this example are listed in Table 2.

*E. coli* and *P. aeruginosa* were routinely grown in Luria-Bertani (LB) broth or LB agar (Sambrook, *et al.* (1989) *Molecular Cloning: a Laboratory Manual.* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY)), supplemented with antimicrobial agents when necessary. The antimicrobial agents were used at the following concentrations: $HgCl_2$, 15 μg/ml in agar and 7.5 μg/ml in broth; nalidixic acid 20 μg/ml; carbenicillin, 300 μg/ml; tetracycline, 50 μg/ml for *P. aeruginosa* and 20 μg/ml for *E. coli*; and gentamicin, 100 μg/ml for *P. aeruginosa* and 15 μg/ml for *E. coli*. Synthetic acyl-HSL concentrations were 2 μM for $3OC_{12}$-HSL and 5 μM for $C_4$-HSL, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was used at 50 μg/ml.

**DNA Manipulations and Plasmid Constructions**. DNA treatment with modifying enzymes and restriction endonucleases, ligation of DNA fragments with T4 ligase, and transformation of *E. coli* were performed according to standard methods (Ausubel, F. *et al.* (1997) *Short Protocols in Molecular Biology.* (John Wiley & Sons, Inc., New York, N.Y.)). Plasmid isolation was performed using QIAprep spin miniprep kits (Qiagen Inc.) and DNA fragments were excised and purified from agarose gels using GeneClean spin kits (Bio101 Corp.). DNA was sequenced at the University of Iowa DNA core facility by using standard automated sequencing technology.

To construct pMW10, the pBR322 *tetA(C)* gene-containing *Cla*I-*Not*I DNA fragment in pJPP4 was replaced with a *tetA(B)*-containing *Bst*B1-*Not*I fragment from Tn10. It was necessary to use *tetA(B)* rather than *tetA(C)* to inactivate *lasI* because the *tetA(C)* gene from pBR322 was a hot spot for Tn5::B22 mutagenesis (Berg, D. E. *et al.* (1983) *Genetics* 105, 813-828).

To construct pMW300 a 1.6-kb *Sma*I fragment from pGMΩ1 that contained the *aacC1* gene (encoding gentamicin acetyltransferase-3-1) was cloned into *Eag*I digested pTL61T, which had been polished with T4 polymerase. The resulting plasmid pTL61T-GMΩ1 was digested with *Sma*I and *Msc*I to release a 6.5-kb *lacZ-aacC1* fragment. A

TABLE 2. Bacterial strains and plasmids

| Strain or plasmid | Relevant characteristics | Source (reference) |
|---|---|---|
| Strains | | |
| *P. aeruginosa* PAO1 | Parental strain | (1) |
| *P. aeruginosa* PDO100 | Δ*rhlI*::Tn501 derivative of PAO1, Hg$^r$ | (2) |
| *P. aeruginosa* PAO-MW1 | Δ*lasI*, Δ*rhlI* derivative of PDO100, Hg$^r$, Tc$^r$ | This study |
| *P. aeruginosa* PAO-MW10 | *lasB*::*lacZ* chromosomal insertion in PAO-MW1 | This study |
| *E. coli* DH5α | F$^-$ φ80Δ*lacZ*, Δ*M15*, Δ(*lacZYA-argF*)U169, *endA1*, *recA1*, *hsdR17*, *deoR*, *gyrA96*, *thi-1* *relA1*, *supE44* | (3) |
| *E. coli* HB101 | F$^-$ *mcrB*, *mrr hsdS20*, *recA13*, *leuB6*, *ara-14* *proA2*, *lacY1*, *galK2*, *xyl-5*, *mtl-1*, *rpsL20* (Sm$^r$), *supE44* | (3) |
| *E. coli* SY327 λpir | (λpir), Δ(*lac pro*), *argE*(Am), *rif*, *nlA*, *recA56* | (4) |
| *E. coli* S17-1 | *thi*, *pro*, *hsdR*, *recA*, *RP4-2* (Tet::Mu) (Km::Tn7) | (5) |
| Plasmids | | |
| pJPP4 | oriR6K, mobRP4, Δ*lasI*, Tc$^r$, Ap$^r$ | (6) |
| pTL61T | *lacZ* transcriptional fusion vector, Ap$^r$ | (7) |
| pGMΩ1 | Contains *aacI* flanked by transcriptional and translational stops, Gm$^r$ | (8) |
| pTL61T-GMΩ1 | pTL61T with *aacI* gene from pGMΩ1 upstream of *lacZ*, Ap$^r$, Gm$^r$ | This study |
| pMW100 | pJPP4 with 2.7-kb *tet*A(B) from Tn10 in place of the pBR322 *tetA(C)*, Tc$^r$, Ap$^r$ | This study |
| pRK2013 | ori (ColE1), *tra*$^-$, (RK2)Km$^r$ | (9) |
| pSUP102 | pACYC184 carrying mobRP4, Cm$^r$, Tc$^r$ | (10) |
| pSUP102-lasB | pSUP102 carrying *lasB* on a 3.1-kb *P. aeruginosa* chromosomal DNA fragment, Cm$^r$, Tc$^r$ | This study |
| pMW300 | pSUP102-*lasB* containing *lacZ-aacI* from pTL61T-GMΩ1 (*lasB-lacZ* transcriptional fusion knockout plasmid), Cm$^r$, Gm$^r$ | This study |
| pTn5-B22 | pSUP102 with Tn5-B22 ('*lacZ*), Gm$^r$ | (28) |

Abbreviations for antibiotics are as follows: kanamycin, Km; gentamicin, Gm; ampicillin, Ap; tetracycline, Tc; streptomycin, Sm.

3.1-kb *P. aeruginosa* PAO1 chromosomal DNA fragment containing the *lasB* gene was amplified by PCR using the Expand™ Long Template PCR System (Boehringer

Mannheim). This fragment was cloned into *Bam*HI-digested pSUP102. The resulting plasmid, pSUP102-lasB was digested with *Not*I, polished with T4 polymerase and ligated with the 6.5-kb *lacZ-aacC1* fragment from pTL61T-GMΩ1 to generate pMW300. The promoterless *lacZ* gene in pMW300 is 549 nucleotides form the start of the *lasB* ORF, it is flanked by 1.5 kb upstream and 1.6 kb downstream *P. aeruginosa* DNA, and it contains the p15A *ori*, which does not support replication in *P. aeruginosa*.

**Construction of *P. aeruginosa* Mutants.** A *lasI, rhlI* mutant strain of *P. aeruginosa* PAO-MW1 was generated by insertional mutagenesis of *lasI* in the *rhlI* deletion mutant, PDO100. For insertional mutagenesis, the *lasI::tetA(B)* plasmid, pMW100 was mobilized from *E. coli* SY327 *λpir* into PDO100 by triparental mating with the help of *E. coli* HB101 containing pRK2013. Because pMW100 has a *λpir*-dependent origin of replication, it cannot replicate in *P. aeruginosa*. A tetracycline-resistant, carbenicillin-sensitive exconjugant was selected, which was shown by a Southern blot analysis to contain *lasI:tetA* but not *lasI* or pMW100. To confirm the inactivation of the chromosomal *lasI* in this strain, PAO-MW1, the amount of $3OC_{12}$-HSL in the fluid from a stationary phase culture (optical density at 600 nm, 5) was assessed by a standard bioassay (Pearson, J. P. *et al.* (1994) *PNAS*. **91,** 197-201). No detectable $3\text{-}OC_{12}$-HSL (< 5 nM) was found.

A mutant strain, *P. aeruginosa* PAO-MW10, which contains a *lacZ* reporter in the chromosomal *lasB* gene was constructed by introduction of pMW300 into PAO-MW1 by triparental mating as described above. Exconjugants resistant to gentamicin and sensitive to chloramphenicol were selected as potential recombinants. Southern blotting of chromosomal DNA with *lasB* and *lacZ* probes indicated that the pMW300 *lasB-lacZ* insertion had replaced the wt *lasB* gene.

**Southern Blotting.** Chromosomal DNA was prepared using the QIAMP tissue kit (Qiagen Inc.). Approximately 2 μg of chromosomal DNA was digested with restriction endonucleases, separated on a 0.7% agarose gel, and transferred to a nylon membrane according to standard methods (Ausubel, F. *et al.* (1997) *Short Protocols in Molecular Biology*. (John Wiley & Sons, Inc., New York, N.Y.). DNA probes were generated using digoxigenin-11-dUTP by random primed DNA labeling or PCR. The

Southern blots were visualized using the Genius™ system as outlined by the manufacturer (Boehringer Mannheim).

**Tn5 Mutagenesis.** Tn5::B22, which carries a promoterless *lacZ* gene, was used to mutagenize *P. aeruginosa* PAO-MW1 (Simon, R. *et al.* (1989) *Gene* 80, 161-169). Equal volumes of a late logarithmic phase culture of *E. coli* S17-1 carrying pTn5::B22 grown at 30°C with shaking and a late logarithmic phase culture of *P. aeruginosa* PAO-MW1 grown at 42°C without shaking were mixed. The mixture was centrifuged at 6000 x *g* for 10 minutes at room temperature, suspended in LB (5% of the original volume), and spread onto LB plates (100 μl per plate). After 16 to 24 hours at 30°C, the cells on each plate were suspended in 500 μl LB and 100 μl volumes were spread onto LB agar plates containing $HgCl_2$, gentamicin, tetracycline and nalidixic acid. The nalidixic acid prevents growth of *E. coli* but not *P. aeruginosa*. After 48 to 72 hours at 30°C, 20 colonies were selected from each mating and grown on LB selection agar plates containing X-gal. Ten of the 20 were picked for further study. The colonies picked showed a range in the intensity of the blue color on the X-gal plates. In this way, the selection of siblings in a mating were minimized. A Southern blot using a probe to *lacZ* was performed on 20 randomly chosen transconjugants indicated that the Tn5 insertion in each was in a unique location.

**The Screen for qsc Fusions.** A microtiter dish assay was used to identify mutants showing acyl-HSL-dependent β-galactosidase expression (quorum sensing-controlled or qsc mutants). Each transconjugant was grown in four separate wells containing LB broth without added autoinducer, with added $3OC_{12}$-HSL, $C_4$-HSL, or both $3OC_{12}$-HSL and $C_4$-HSL for 12-16 hours at 37°C. Inocula were 10 μl of an overnight culture and final culture volumes were 70 μl. The β-galactosidase activity of cells in each microtiter dish well was measured in microtiter dishes with a luminescence assay (Tropix) Luminescence was measured with a Lucy I microtiter dish luminometer (Anthos).

**Patterns of Acyl-HSL Induction of β-galactosidase Activity in qsc Mutants.** The pattern of β-galactosidase expression was examined in response to acyl-HSLs in each of 47 qsc mutants identified in the initial screen. Each mutant was grown in 1 ml of MOPS (50 mM, pH 7.0) buffered LB broth containing one, the other, both, or neither acyl-HSL signal in an 18 mm culture tube at 37°C with shaking. A mid-logarithmic phase culture was used as an inoculum and initial optical densities (ODs) at 600 nm were 0.1. Growth was monitored as OD at 600 nm and β-galactosidase activity was measured in 0.1 ml samples taken at 0, 2, 5, and 9 hours after inoculation.

**DNA Sequencing and Sequence Analysis.** To identify DNA sequences flanking Tn5::B22 insertions, arbitrary PCR was performed with primers and conditions as described (Caetano-Annoles, G. (1993) *PCR Methods Appl.* **3**, 85-92; O'Toole, G. A. *et al.* (1998) *Mol. Microbiol.* 28, 449-461). Tn5 flanking sequences that could not be identified using arbitrary PCR were cloned. For cloning, 3 μg of chromosomal DNA was digested with *Eco*RI and ligated with *Eco*RI-digested, phosphatase treated pBR322. *E. coli* DH5α was transformed by electroporation with the ligation mixtures and plasmids from gentamicin resistant colonies were used for sequencing Tn5-flanking DNA.

DNA sequences flanking Tn5-B22 insertions were located on the *P. aeruginosa* PAO1 chromosome by searching the chromosomal database at the *P. aeruginosa* Genome Project web site (www.pseudomonas.com). The ORFs containing the insertions are those described at the web site. Functional coupling from the Argonne National Labs (http://wit.mcs.anl.gov/WIT2), sequence analysis, and expression patterns of the qsc mutants were used to identify potential operons (Overbeek, R. *et al.* (1999) *PNAS* 96, 2896-2901).

## Results

**Identification of *Pseudomonas aeruginosa* qsc Genes.** Seven thousand Tn5::B22 mutants of *P. aeruginosa* PAO-MW1 were screened. Tn5::B22 contains a promoterless *lacZ*. *P. aeruginosa* PAO-MW1 is a *lasI, rhlI* mutant that does not make acyl-HSL signals. Thus, transcription of the Tn5::B22 *lacZ* in a qsc gene was expected to respond to an acyl-HSL signal. The screen involved growth of each mutant in a

complex medium in a microtiter dish well with no added acyl-HSL, $3OC_{12}$-HSL, $C_4$-HSL, or both $3OC_{12}$-HSL and $C_4$-HSL. After 12-16 hours, β-galactosidase activity in each culture was measured. Two hundred-seventy mutants showed greater than2 fold stimulation of β-galactosidase activity in response to either or both acyl-HSL. Of these, 70 showed a greater than5-fold stimulation of β-galactosidase activity in response to either or both acyl-HSL, and were studied further. Each mutant was grown with shaking in culture tubes and 47 showed a reproducible greater than5-fold stimulation of β-galactosidase activity in response to either or both of the acyl-HSL signals. These were considered to have Tn5::B22 insertions in qsc genes. It was shown by a Southern blot analysis with a *lacZ* probe that each mutant contained a single Tn5::B22 insertion.

This collection of 47 mutants is not believed to represent the entire set of quorum sensing regulated genes in *P. aeruginosa*. The threshold of greater than5-fold induction may be too stringent, enough mutants may not have been screened to be assure that insertions in all of the genes in the chromosome have been tested, and there may be conditions other than those which were employed that would have revealed other genes which were not detected in the present screen. Nevertheless, a set of 47 insertions in genes have been identified that show significant activation in response to acyl-HSL (qsc genes).

**Responses of qsc Mutants to Acyl-HSL Signals.** For cultures of each of the 47 qsc mutants, β-galactosidase activity was measured at different times after addition of acyl-HSL signals. The basal levels of β-galactosidase varied depending on the mutant. The responses to the acyl-HSL signals could be grouped into 4 general classes based on which of the two signals was required for activation of *lacZ*, and whether the response to the signal(s) occurred immediately or was delayed until stationary phase. A response was considered immediate if there was a 5-fold or greater response within 2 hours of acyl-HSL addition (the optical densities(ODs) of the cultures ranged from 0.5-0.7 at 2 hours). A response was considered delayed or late if there was <5-fold induction at 2 hours but greater than5-fold induction of β-galactosidase at 5 hours or later (ODs of 2 or greater). In some strains activation of *lacZ* required $3OC_{12}$-HSL, others required both $3OC_{12}$-HSL and $C_4$-HSL for full activation of *lacZ*. A number of strains responded to either signal alone but showed a much greater response with both $3OC_{12}$-HSL and $C_4$-

HSL. None of the mutants responded well to $C_4$-HSL alone (Table 3). This was expected because expression of RhlR, which is required for a response to $C_4$-HSL is dependent on $3OC_{12}$-HSL (Pesci, E. C. *et al.* (1997) *J. Bacteriol.* 179, 3127-3132). Therefore at least some of the insertions exhibiting a response to both acyl-HSLs may be responding to the *rhl* system, which requires activation by the *las* system.

Class I mutants responded to $3OC_{12}$-HSL immediately, Class II responded to $3OC_{12}$-HSL late, Class III respond best to both signals early, and Class IV to both signals late. There were 9 Class I, 11 Class II, 18 Class III, and 9 Class IV mutants. Figure 2 shows responses of representative members of each class to acyl-HSLs. Generally, most early genes (Class I and III genes) showed a much greater induction than most late genes (Class II and IV). Many of the Class III mutants showed some response to either signal alone but showed a greater response in the presence of both signals (Table 3 and Figure 2).

**Identity and Analysis of qsc Genes.** The Tn5-B22-marked qsc genes were identified by coupling arbitrary PCR or transposon cloning with DNA sequencing. The sequences were located in the *P. aeruginosa* PAO1 chromosome by searching the *Pseudomonas aeruginosa* Genome Project web site (www.pseudomonas.com). To confirm the locations of the Tn5-B22 insertions in each qsc mutant, a Southern blot analysis was performed with Tn5-B22 as a probe. The sizes of Tn5-B22 restriction fragments were in agreement with those predicted based on the *P. aeruginosa* genomic DNA sequence (data not shown). The 47 qsc mutations mapped in or adjacent to 39 different open reading frames (ORFs). For example Figure 3 depicts the nucleic acid sequence of the quorum sensing controlled locus on the *P. aeruginosa* chromosome mapped in the *P. aeruginosa* mutant strain qsc102.

**Table 3.** Quorum sensing-controlled genes in *Pseudomonas aeruginosa*

| Classification | Identity[a] | Signal response[b] 3OC$_{12}$-HSL | $C_4$-HSL | Both | Genomic Position[e] |
|---|---|---|---|---|---|
| Class I | | | | | |
| qsc100 | Peptide synthetase | 65 | 3 | 69 | 5801998 |
| qsc101 | No match | 145 | 1 | 184 | 7730 |
| qsc102 | No match | 350 | 1 | 400 | 5547 |
| qsc103 | No match | 85 | 1 | 95 | 3961920 |
| qsc104 | Polyamine binding protein | 7 | 2 | 8 | 5402505 |
| qsc105 | FAD-binding protein | 40 | 1 | 42 | 5410045 |
| qsc106A&B | No match | 9 | 1 | 10 | 2870317 |
| qsc107 | No match | 44 | 2 | 50 | 5799641 |
| Class II | | | | | |
| qsc108 | **ORF 5** | 13 | 1 | 7 | 5617382 |
| qsc109 | Bacitracin synthetase 3 | 13 | 1 | 8 | 5651872 |
| qsc110A&B | Pyoverdine synthetase D | 10 | 1 | 7 | 5661697 |
| qsc111 | Pyoverdine synthetase D | 11 | 1 | 7 | 5666282 |
| qsc112A&B | Aculeacin A acylase | 15 | 1 | 12 | 5701004 |
| qsc113 | Trransmembrane protein | 5 | 1 | 5 | 3771157 |
| qsc114[c] | No match | 9 | 1 | 7 | 5209051 |
| qsc115[d] | No match | 4 | 1 | 5 | 1941557 |
| qsc116 | No match | 5 | 1 | 5 | 1138940 |
| Class III | | | | | |
| qsc117[d] | **ACP-like protein** | 22 | 22 | 186 | 41430 |
| qsc118 | **RhlI** | 38 | 14 | 70 | 4447967 |
| qsc119 | **RhlB** | 9 | 7 | 100 | 4446918 |
| qsc120 | Chloramphenicol resistance | 3 | 7 | 24 | 4592102 |
| qsc121 | 3-Oxoacyl ACP synthase | 13 | 27 | 105 | 4594988 |
| qsc122A&B | Cytochrome p450 | 2 | 10 | 90 | 4595538 |
| qsc123 | 9-Cis retinol dehydrogenase | 14 | 28 | 96 | 4597340 |
| qsc124A&B | Pyoverdine synthetase D | 35 | 50 | 148 | 4598281 |
| qsc125 | Zeaxanthin synthesis | 20 | 65 | 140 | 4600099 |
| qsc126 | Pristanimycin I synthase 3 & 4 | 3 | 5 | 24 | 4603518 |
| qsc127[c] | No match | 5 | 2 | 15 | 4608787 |
| qsc128 | Hydrogen cyanide synthase HcnB | 19 | 12 | 42 | 5924799 |
| qsc129A&B | Cellulose binding protein p40 | 15 | 1 | 100 | 1141723 |
| qsc130 | *glc* operon transcriptional activator | 5 | 1 | 14 | 2313744 |
| qsc131 | **PhzC** | 50 | 168 | 742 | 1110 |

| | | | | | |
|---|---|---|---|---|---|
| Class IV | | | | | |
| qsc132A&B | Unknown (*B. pertusis*) | 1 | 1 | 40 | 3616599 |
| qsc133A&B | AcrB | 1 | 1 | 9 | 3628342 |
| qsc134 | Saframycin Mx1 synthetase A | 6 | 1 | 28 | 3781254 |
| qsc135 | **Cytochrome C precursor** | 3 | 1 | 6 | 4942182 |
| qsc136[c] | No match | 7 | 3 | 45 | 851491 |
| qsc137 | Asparagine synthetase | 1 | 1 | 10 | 2007007 |
| qsc138 | No match | 3 | 5 | 32 | 2459178 |

[a] The bold letters indicate matches were to known *P. aeruginosa* genes.

[b] The signal response is given as β-galactosidase activity in cells grown in the presence of the indicated signal(s) divided by the β-galactosidase activity of cells grown in the absence of added signals. Maximum responses are indicated.

[c] The *lacZ* insertions in these strains are in the opposite orientation of the ORFs described in the *P. aeruginosa* Genome Project web site. The insertions are which in locations with no reported identity are been indicated.

[d] Insertions do not lie in but are near the putative ORFs indicated. In qsc117 the insertion is 129 bp downstream of the ACP ORF and interrupts a potential rho-independent transcription terminator. The qsc115 insertion is 60 bp upstream of the ORF listed in Materials and Methods.

[e] Genomic position as identified using sequence information described in the *P. aeruginosa* Genome Project web site (July 15. 1999 release).

The genomic sequences comprising the ORFs in Table 3 are described in the *Pseudomonas aeruginosa* Genome Sequencing Project web site, as detailed in Table 4.

Only 2 genes were identified that already were known to be controlled by quorum sensing, *rhlI* and *rhlB*. Several other genes potentially involved in processes known to be regulated by quorum sensing were also identified including *phzC* (phenazine synthesis), a putative cyanide synthesis gene (related to the *Pseudomonas fluorescens hcnB* ), and ORF 5 (pyoverdine synthesis) (Latifi, A. *et al.* (1995) *Mol. Microbiol.* 17, 333-344; Cunliffe, H. E. *et al.* (1995) *J. Bacteriol.* 177, 2744-2750). Interestingly, *lasB* was not identified by the assay, yet the LasI-LasR quorum sensing system was originally described as regulating *lasB* (Gambello, M. J. *et al.* (1991) *J. Bacteriol.* 173, 3000-3009). A *lasB-lacZ* chromosomal fusion in *P. aeruginosa* PAO-MW1 was constructed, so that regulation of *lasB* by quorum sensing could be compared to the genes identified by the assay. The *lasB-lacZ* fusion only responded slightly to $3OC_{12}$-HSL (3-fold stimulation). The full response (12-13-fold over background)

required both $C_4$-HSL and $3OC_{12}$-HSL, and the response was late (Figure 2). Thus, *lasB* shows the characteristics of a Class IV gene.

Some of the qsc mutants had obvious phenotypes. Unlike the parent, on LB agar, colonies of the Class II mutants qsc108, 109, 110A and B, and 111 were not fluorescent. Because pyoverdine is a fluorescent pigment, and because the qsc110 and 111 mutations were in genes coding for pyoverdine synthetase-like proteins, it was suspected that these mutations define a region involved in pyoverdine synthesis. The insertion in qsc131 is in *phzC* which is required for pyocyanin synthesis. Although the parent strain produced a blue pigment in LB broth, qsc131 did not. The two qsc132 mutants also did not produce detectable levels of pyocyanin but did produce a water-soluble red pigment.

Functional coupling and sequence analysis were used to identify 7 putative qsc operons, one of which is the previously described *rhlAB* operon (Figure 4). Functional coupling will not organize genes encoding polypeptides without known relatives into operons, and organization of genes in an operon was disallowed in cases where there was greater than 250 bp of intervening sequence between two adjacent ORFs. The

**Table 4.** ORFs of quorum sensing-controlled genes in *Pseudomonas aeruginosa*

| QSC | Insertion July 15. 1999 release | Insertion December 15. 1999 release | Open Reading Frame December 15. 1999 release | Orientation | SEQ ID NO |
|---|---|---|---|---|---|
| 131 | 1110 | 4715256 | 4714774-4715991 | Forward | 1 |
| 102 | 5547 | 2067716 | 2066736-2068517 | Reverse | 2 |
| 101 | 7730 | 2065297 | 2064803-2065495 | Reverse | 3 |
| 117 | 41430 | 2031833 | 2031245-2031655 | Forward | 4 |
| 136 | 851491 | 1221771 | 1221374-1221961 | Reverse | 5 |
| 116 | 1138940 | 934322 | 934191-935210 | Reverse | 6 |
| 129 | 1141723 | 931539 | 930603-931772 | Reverse | 7 |
| 115 | 1941557 | 131753 | 131583-131792 | Reverse | 8 |
| 137 | 2007007 | 66507 | 66264-68135 | Forward | 9 |
| 130 | 2313744 | 6023975 | 6023787-6024542 | Forward | 10 |
| 138 | 2459178 | 5878418 | 5877776-5878597 | Forward | 11 |
| 106 | 2870317 | 5467402 | 5466520-5467887 | Forward | 12 |
| 132 | 3616599 | 4721118 | 4720249-4721457 | Forward | 13 |
| 133 | 3628342 | 4709375 | 4707483-4710572 | Forward | 14 |
| 113 | 3771157 | 4566558 | 4565369-4567903 | Reverse | 15 |
| 134 | 3781254 | 4556461 | 4555202-4558177 | Forward | 16 |
| 103 | 3961920 | 4375793 | 4375589-4376680 | Forward | 17 |
| 119 | 4446918 | 3890793 | 3890724-3892004 | Reverse | 18 |
| 118 | 4447967 | 3889744 | 3889088-3889738 | Reverse | 19 |
| 120 | 4592102 | 3745609 | 3744850-3746016 | Forward | 20 |
| 121 | 4594988 | 3742723 | 3742643-3743635 | Forward | 21 |
| 122 | 4595538 | 3742173 | 3740961-3742217 | Forward | 22 |
| 123 | 4597340 | 3740171 | 3740054-3740968 | Forward | 23 |
| 124 | 4598281 | 3739430 | 3738724-3740052 | Forward | 24 |
| 125 | 4600099 | 3737612 | 3737561-3738727 | Forward | 25 |
| 126 | 4603518 | 3734193 | 3730455-3737564 | Forward | 26 |
| 127 | 4608787 | 3728924 | | Reverse | |
| 135 | 4942182 | 3395532 | 3395274-3396677 | Reverse | 27 |
| 114 | 5209051 | 3128663 | 3127731-3129116 | Forward | 28 |
| 104 | 5402505 | 2935208 | 2934490-2935593 | Forward | 29 |
| 105 | 5410045 | 2927668 | 2926722-2927972 | Reverse | 30 |
| 108 | 5617382 | 2720329 | 2718890-2720643 | Reverse | 31 |
| 109 | 5651872 | 2678258 | 2671678-2679012 | Reverse | 32 |
| 110 | 5661697 | 2676014 | 2671678-2679012 | Reverse | 32 |
| 111 | 5666282 | 2671429 | 2669119-2671674 | Reverse | 33 |
| 112 | 5701004 | 2636707 | 2636467-2638800 | Reverse | 34 |
| 107 | 5799641 | 2538070 | 2532619-2539008 | Reverse | 35 |

| | | | | | |
|---|---|---|---|---|---|
| 100 | 5801998 | 2535711 | 2532619-2539008 | Reverse | 35 |
| 128 | 5924799 | 2412909 | 2412807-2414201 | Forward | 36 |

qsc101 and 102 genes are an example of a putative operon that was not identified by functional coupling (Figure 4). These two ORFs did not show significant similarities with other polypeptides. Nevertheless, they are transcribed in the same direction, closely juxtaposed, qsc101 and 102 are both Class I genes, and there is a *las* box-like element upstream of these ORFs. Expression of the qsc102 insertion is controlled by an upstream ORF (SEQ ID NO:37) which comprises the sequences between postions 2068711 to 267911 of the *P. aeruginosa* genome (December 15, 1999 release) which in turn is preceded by a *las* box regulatory element (SEQ ID NO:38) which comprises the sequences between postions 2068965 to 2068946 of the *P. aeruginosa* genome (December 15, 1999 release). The *las* box is a palindromic sequence found upstream of and involved in LasR-dependent activation of *lasB* (Rust, L. *et al.*, (1996) *J. Bacteriol.* 178, 1134-1140).

The qsc133A and B insertions are in a putative 3-gene operon with similarity to *acrAB-tol*C from *E. coli* and the *mex-opr* family of efflux pump operons in *P. aeruginosa*, one of which (*mexAB-oprN*) has been shown to aid $3OC_{12}$-HSL efflux (Kohler, T., *et al.* (1997) *Mol. Microbiol.* 23, 345-354; Poole, K, *et al.* (1993) *J. Bacteriol.* 175, 7363-7372; Poole, K.*et al.* (1996) *Mol. Microbiol.* 21, 713-724; Evans, K., *et al.* (1998) *J. Bacteriol.* 180, 5443-5447; Pearson, J. P. *et al.* (1999) *J. Bacteriol.* 181, 1203-1210). The qsc133 mutations are within a gene encoding a MexF homolog. The qsc133 mutants show typical Class IV regulation. Expression of *lacZ* is late and dependent on the presence of both acyl-HSL signals (Table 3 and Figure 2). No *las* box-like sequences upstream of this suspected efflux pump operon were identified.

A third possible operon identified by functional coupling is about 8 kb and contains 10 genes. Eight strains with insertions in 6 of the 10 genes were obtained, all of which are Class III mutants (Table 3). A *las* box-like sequence was identified upstream of the first gene of this operon. The function of these 10 genes is unknown but the similarities shown in Table 2 suggest that they may encode functions for synthesis and resistance to an antibiotic-like compound.

The qsc128 mutation is within a gene coding for a polypeptide that shows similarity to the *P. fluorescens hcnB* product and appears to be in a 3-gene operon (Table 3, Figure 4). By analogy to the *P. fluorescens hcn* operon, this operon is likely required for the production of the secondary metabolite, hydrogen cyanide. Previous investigations have shown that hydrogen cyanide production is reduced in *P. aeruginosa rhl* quorum sensing mutants. Consistent with this, qsc128 is a Class III mutant (Table 2). Full induction required both acyl-HSL signals, however, some induction of *lacZ* resulted from the addition of either signal alone (Table 3). A *las* box-like sequence was identified in the region upstream of the translational start codon of the first gene in this operon. This *las*-type box may facilitate an interaction with either LasR or RhlR.

The *phz* operon, required for phenazine biosynthesis, has been described in other pseudomonads and the insertion in strain qsc131 is located in a gene encoding a *phzC* homolog. Analysis of the sequence around this *phzC* homolog revealed an entire phenazine biosynthesis operon, *phzA-G* (Georgakopoulos, D. G. *et al.* (1994) *Appl. Environ. Microbiol.* 60, 2931-2938; Mavrodi, D. V. *et al.* (1998) *J. Bacteriol.* 180, 2541-2548). As discussed above, qsc131 does not produce the blue phenazine pigment pyocyanin. PhzC is part of an operon of several genes including PhzABCDEFG, and transcription of this operon is controlled by the promoter region (SEQ ID NO:39) in front of the first gene in the operon, PhzA. The *phz* operon in *P. aeruginosa* also contains a *las*-box like sequence upstream of the first gene of the operon. The PhzA promoter region (SEQ ID NO:39) has been cloned into a plasmid, transcriptionally fused to lacZ. The resulting plasmid (pMW303G) was transformed into PAO1 and used as a reporter strain. The resultant bacterial strain generates a quorum sensing signal and responds to it by increased β-galactosidase activity. As shown in Figure 5, this strain displayed a high level of induction between early and late growth, thus providing a dynamic range for detecting modulation (*e.g.*, inhibition) of quorum sensing signaling. Accordingly this strain may be useful for a single strain assay for identifying for inhibitors of quorum sensing singaling, as described herein.

The final putative operon consists of 2 or 3 genes, qsc109-111, which appear to be involved in pyoverdine synthesis. These ORFs were not identified in the *P. aeruginosa* genome project web site but were identified and shown to be functionally coupled with the Argonne National Laboratory web site.

For three of the qsc insertions, the *lacZ* gene was in an orientation opposite to the ORF described in the Genome Project web site (qsc114, 127, and 136).

**Locations of qsc Genes on the *P. aeruginosa* Chromosome.** The qsc genes were mapped to sites on the *P. aeruginosa* chromosome (Figure 6). In addition *lasB*, *lasR* and *lasI*, and *rhlR* were placed on this map. The distribution of currently identified qsc genes is patchy. For example, 16 of the 39 qsc genes representing 3 of the classes mapped to a 600-kb region of the 6 megabase chromosome. A 140-kb island of 12 Class III genes, 8 transcribed in one direction and 4 transcribed in the other direction (including the *rhl* genes) formed another cluster on the chromosome.

**Identification of *las* Box-Like Sequences that Could be Involved in qsc Gene Control.** As discussed above, the *las* box is a palindromic sequence found upstream of and involved in LasR-dependent activation of *lasB* (Rust, L. *et al.* (1996) *J. Bacteriol.* 178, 1134-1140). The *las* box shows similarity to the *lux* box, which is the promoter element required for quorum control of the *V. fischeri* luminescence genes (Devine, J. *et al.* (1989) *PNAS* 86, 5688-5692). Elements similar to a *las* box were identified by searching upstream of qsc ORFs. A search was done for sequences with at least 50% identity to the *las* box found 42 bp upstream of the *lasB* transcriptional start site (Rust, L. *et al.* (1996) *J. Bacteriol.* 178, 1134-1140). *las* box-like sequences were identified which are suspected to be involved in the regulation of 14 of the 39 qsc genes listed in Table 1 (Figure 7). Because there is little information on the transcription starts of most of the genes identified in the screening assay, some relevant *las* boxes may have been missed and some of the identified sequences may not be in relevant positions.

## Discussion

By screening a library of *lacZ* promoter probes introduced into *P. aeruginosa* PAO1 by transposon mutagenesis, 39 quorum sensing controlled (qsc) genes were identified. Most of these genes were not identified as quorum sensing-controlled previously. Mutations were not found in every gene in putative qsc operons (Figure 4). Mutants that showed only a small degree of acyl-HSL-dependent *lacZ* induction in the initial screen were not studied. Thus, it is presumed that all of the quorum sensing

controlled (qsc) genes have been identified. A conservative estimate is that about 1% of the genes in *P. aeruginosa* are controlled by quorum sensing (39 out of about 5,000-6,000 genes in the *P. aeruginosa* chromosome were confirmed to be qsc without saturating the mutagenesis). A more liberal estimation of 3-4% can be drawn from the finding of 270 mutants showing at least a 2-fold induction in response to one or both of the acyl-HSL signals in the initial screen of 7,000 mutants.

Several mutants, for example qsc101 and 102 showed an immediate and relatively large response to $3OC_{12}$-HSL (Class I mutants, Table 3). The qsc101 and 102 genes code for proteins with no matches in the databases. Several mutants showed a relatively large and immediate response when both signals were supplied in the growth medium. Examples are qsc119 (*rhlB*), 121-125, and 129A and B. The qsc mutant showing the largest response was qsc131. The level of β-galactosidase activity when this mutant was grown in the presence of both signals was greater than700 times that in the absence of the signals (Table 3). The qsc131 mutation is in *phzC*, which is a phenazine biosynthesis gene, and the qsc131 mutant did not produce the blue phenazine pigment pyocyanin at detectable levels. Many of the mutants that responded best to both signals early (Class III mutants) showed a small response when exposed to one or the other signal. The reasons for the small response to either signal are unclear at present but the data suggest that these genes may be subject to signal cross talk, or they may show a response to either LasR or RhlR. One reason they may respond to both signals better than they respond to $C_4$-HSL alone is that $3OC_{12}$-HSL and LasR are required to activate RhlR, the transcription factor required for a response to $C_4$-HSL (Latifi, A. *et al.* (1996) *Mol. Microbiol.* 21, 1137-1146; Pesci, E. C. *et al.* (1997) *J. Bacteriol.* 179, 3127-3132). There were two mutant classes that showed a delayed response to the signals; Class II mutants which required only $3OC_{12}$-HSL, and Class IV mutants, which required both signals for full induction. These mutants showed between 5 and 45-fold activation of gene expression (Table 3). There are a number of possible explanations for a delayed response to signal addition. It is possible that some of these genes are stationary phase genes. It is also possible that some are iron repressed. For example, it is known that the synthesis of pyoverdine is regulated by iron and the Class II, delayed response, qsc108-111 mutations are in genes involved in pyoverdine synthesis (Cunliffe, H. E. *et al.* (1995) *J. Bacteriol.* 177, 2744-2750; Rombel, I. *et al.* (1995) *Mol. Gen. Genet.* 246,

519-528). It is also possible that some of these genes are not regulated by quorum sensing, directly. The acyl-HSL signals might control other factors that influence expression of any of the genes that have been identified and this possibility seems most likely with the late genes in Classes II and IV. Indirect regulation may not be common for late genes. This is known because the *lasB-lacZ* chromosomal insertion which was generated by site-specific mutation was in Class IV, and it is known from other investigations that *lasB* responds to LasR and $3OC_{12}$-HSL, directly (Passador, L. *et al.* (1993) *Science* 260, 1127-1130; Rust, L. *et al.* (1996) *J. Bacteriol.* 178, 1134-1140). The two classes of late qsc genes may be comprised of several subclasses.

*Las* boxes are genetic elements which may be involved in the regulation of qsc genes. Although sequences with characteristics similar to *las* boxes were identified, (Figure 7), the locations of these sequences have not provided insights about the differences in the patterns of gene expression among the four classes of genes. It is possible that when the promoter regions of the qsc genes are studied that particular motifs in the regulatory DNA of different classes of genes will be revealed.

Many of the qsc genes appear to be organized in two patches or islands on the *P. aeruginosa* chromosome (Figure 7). Because LasR mutants are defective in virulence it is tempting to speculate that these gene clusters may represent pathogenicity islands. The *rhlI-rhlR* quorum sensing modulation occurs on one of the qsc islands, but none of the qsc genes are tightly linked to the *lasR-lasI* modulon. Genes representing each of the 4 classes occur over the length of the chromosome and on both DNA strands. This is consistent with the view that quorum sensing is a global regulatory system in *P. aeruginosa*. Of interest there is a third LuxR family member in *P. aeruginosa*. This gene is adjacent to and divergently oriented from qsc103.

Quorum sensing is critical for virulence of *P. aeruginosa* and for the development of mature biofilms. The methodology disclosed herein for identification of qsc genes provides a manageable group of genes to test for function in virulence and biofilm development. Furthermore, the availability of the *P. aeruginosa* genome sequence will very likely lead to the development of a gene expression microarray for this organism. The methods described herein provide a set of 39 genes that respond to specific treatments in a predictable fashion (Table 3).

## EXAMPLE 2 SCREENING ASSAY FOR QUORUM SENSING INHIBITING COMPOUNDS

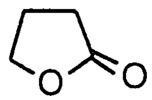
In this example, the screening assay used two strains of *P. aeruginosa*: a wild type *P. aeruginosa* (PAO1) and QSC102, from Example 1 (see Figure 8). This assay will detect inhibition of all aspects of quorum sensing signaling, *e.g.*, signal generation and signal reception.

**Procedural Overview**

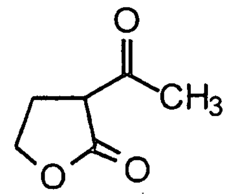
Microtiter plates are prepared by adding 200 μL Luria Broth ("LB") agar, containing 0.008 % 5-bromo-4-chloro-3-indolyl-β-D-galactose (X-gal) to each well. Overnight cultures of PAO1 and QSC102 are subcultured in LB to a starting absorbance at 600 nm ("A600") of 0.05 and grown at 37 °C to an A600 of 1.0. PAO1 is diluted 2.5 x $10^5$-fold in LB and 5 μL of this is applied to the surface of the LB agar in each well. Plates are then dried in a laminar flow hood for 60 minutes. A tenfold dilution of QSC102 in LB is used to inoculate each well using a replicator. Plates are then sealed and incubated at 37 °C for 40 hours. Growth and color development are evaluated visually and the data is recorded with a camera.

The test compound was present in a microtiter well and overlaid with LB agar and 5-bromo-4-chloro-3-indolyl-β-D-galactose (X-gal). Both strains were spotted on the agar in each well. PAO1 emitted the acyl-HSL signal (3-oxo-C12-HSL), to which QSC102 responded by turning blue. QSC102 expressed β-galactosidase only in response to the LasI signal (3-oxo-C12-HSL); the *lacZ* fusion in QSC102 did not respond to the RhlI signal (C4-HSL). Hence, the assay was selective for inhibitors of the Las system. Inhibition of signaling was evaluated qualitatively by the absence or weakening of the blue color development.

The assay was used to test 6 product analogs, two of which showed an inhibitory effect: butyrolactone and acetyl-butyrolactone. Although bacterial growth was not inhibited, the color development was reduced. Color reduction correlated directly with test compound concentration, although relatively high concentrations (~20 mM) were required to suppress color development completely (Figure 9).



butyrolactone



acetyl-butyrolactone

## EXAMPLE 3 DEVELOPMENT OF A *P. AERUGINOSA* STRAIN FOR A HIGH THROUGHPUT SCREENING ASSAY

### A. Construction of Reporter Strain-Chromosomal Insertion of Reporter

A strain for use in high-throughput screening was constructed by inserting the lacZ transcriptional fusion, linked gentamicin resistance marker, and about 2 kb of flanking DNA from strain QSC102 into a mobilizable plasmid (such as pSUP102) as depicted in Figure 10A. Plasmid pSUP102 confers tetracycline resistance and does not replicate in *P. aeruginosa* (Simon, R. *et al.* (1986) *Meth. Enzym.* 118:640-659). The pSUP102-derivative was then transferred into PAO1 by bi- or triparental mating, selecting for gentamicin resistance (Suh, S. J. *et al.* (1999) *J Bacteriol.* 181(13):3890-7). Gentamicin resistant isolates were screened for tetracycline sensitivity (*i.e.*. a double cross-over event has resulted in a chromosomal insertion). Southern blotting was used to confirm the nature of the recombination event and to rule out candidates with more than one insertion. The resultant bacterial strain generates the signal (3-oxo-C12-HSL) and responds to it by increased β-galactosidase activity. A similar strategy is used to create a reporter strain that expresses *gfp* instead of *lacZ*. The initial GFP variant is the stable and bright variant GFPmut2 (Cormack, B. P. *et al.* (1996) *Gene.* 173(1):33-38).

### Procedural Overview of Assay

A culture of PAQ1 reporter strain (carrying the reporter gene lacZ transcriptionally fused to the regulatory sequence of qsc102 in the wildtype background, PAO1) was grown in LB, 100 μg/ml gentamicin overnight, such that the A600 was

around 0.1. The culture was washed in LB twice and used to subculture at a 1:1000 dilution in LB. The subculture was grown in the presence or absence of test compound. Growth was monitored at A600 and expression of ß-galactosidase activity is measured according to the Miller assay (Miller, J. A. (1976) in *Experiments in Molecular Genetics* pp 352-355, Cold Spring Harbor Lab. Press, Plainview, NY).

The reporter strain was tested by growing it in microtiter plates in the presence and absence of known inhibitors of bacterial signaling. Examples of known inhibitors are: acetyl-butyrolactone, butyrolactone, and methylthioadenosine, a product of the synthase reaction that was shown to be inhibitory to the RhlI synthase (Parsek, M. R. *et al.* (1999) *Proc. Natl. Acad. Sci. USA.* 96:4360-4365). Initial characterization of the assay entailed following the optical density (cell growth) in individual sample wells and measuring induction levels at different time points. Figure 10B shows the induction of β-galactosidase as PAQ1 reaches high density, wherein cell growth is measured at 600 nm (closed circles) and expression of β-galactosidase is measured in Miller units (open circles). For GFP fusions, the fluorescence of the culture is determined after excitation at 488 nm.

**B. Construction of Reporter Strain-Reporter on a Plasmid**

The PAO1/pMW303G strain is constructed as described in Example 1 above.

**Procedural Overview of the Assay**

An overnight culture of PAO1/pMW303G was diluted to an A600 of 0.1 in LB, 300 μg/ml carbenicillin. Of this, 50 μL were added to microtiter plate wells and grown at 37 °C, shaking at 250 rpm, in the presence or absence of test compounds. Culture growth was monitored directly in the microtiter plate at 620 nm. Expression of the reporter gene, ß-galactosidase was measured with the Galacton substrate by Tropix as follows. 12A 20 μL aliquot of the culture was added to 70 μL of 1:100 diluted Galacton substrate (Tropix, PE Biosystems, Bedford, MA) and incubated in the dark at room temperature for 60 minutes. The reaction was stopped and light emission was triggered by the addition of 100 μL Accelerator II (Tropix, PE Biosystems, Bedford, MA), and luminescence was read with plate reader (SpectrofluorPlus, Tecan). Timepoints were taken at 5, 8 and 12 minutes.

In either embodiment of the assay (chromosomal insertion of reporter, or reporter on a plasmid), a satisfactory assay shows normal cell growth but reduced β-galactosidase activity or *gfp* expression in the presence of a known signaling inhibitor. Possible problems associated with the use of fluorescence in whole-cell systems are interference by turbidity as cell density increases and the production of pyocyanin and pyoverdine, fluorescent molecules that are excreted by wild type *P. aeruginosa*. However, interference due to endogenous fluorescent pigments may be reduced by using mutants that lack these pigments (Byng, G. S. *et al.* (1979) *J Bacteriol.* 138(3):846-52).

## EXAMPLE 4 SCREENING ASSAY TO DETERMINE INHIBITION OF THE SIGNAL SYNTHASE

An assay was developed to measure inhibition of RhlI activity, based on a previously published enzyme assay for RhlI (Parsek, M. R. *et al.* (1999) *Proc. Natl. Acad. Sci. USA.* 96:4360-4365). It was shown that the substrates for RhlI are S-adenosylmethionine (SAM) and butanoyl-acyl carrier protein (C4-ACP). It is proposed that RhlI can be used as a model enzyme to study inhibition of acyl-HSL synthases. This is based on the observation that TraI from *Agrobacterium tumefaciens* (Moré, M. I. *et al.* (1996) *Science.* 272(5268):1655-8) and LuxI from *Vibrio fischeri* (Schaefer, A. L. *et al.* (1996) *Proc Natl Acad Sci U S A.* 93(18):9505-9), two homologs of RhlI and LasI, that also utilize SAM and the respective acylated-acyl carrier protein as their substrates.

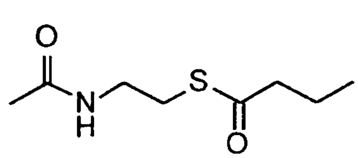
**RhlI activity assay**. Studies of autoinducer synthases have been hampered by the low solubility of the enzyme. It is only in the past year that the first rigorous characterization of an autoinducer synthase was published (Parsek, M. R. *et al.* (1999) *Proc. Natl. Acad. Sci. USA.* 96:4360-4365). This study was performed on RhlI, which had been slightly overproduced in a LasI minus strain of *P. aeruginosa*, thereby avoiding previously encountered problems of solubility. The reaction mechanism deduced for RhlI is summarized in Figure 11. The substrates for the synthase are butanoyl-acyl carrier protein (C4-ACP) and S-adenosylmethionine (SAM). The amino-group of SAM attacks the thioester of C4-ACP to form a peptide bond between butanoic acid and SAM. The first product, acyl carrier protein (ACP) is released. Next, the

SAM-moiety undergoes internal ring closure to form a homoserine lactone (HSL). Methylthioadenosine (MTA) and butanoyl-HSL (C4-HSL) are released.

The enzyme assay reaction mixture contains 60 μM $^{14}C$-labeled SAM and 40 μM C4-ACP in a final volume of 100 μL (buffer: 2 mM dithiothreitol, 200 mM NaCl, 20 mM Tris-HCL, pH 7.8). The reaction is started with the addition of 70 ng RhlI, incubated at 37 °C and quenched after 10 min by addition of 4 μL of 1 M HCl. Product formation is quantitated by extracting the reaction mixtures with 100 μL ethyl acetate and scintillation counting the radiolabeled C4-HSL, which partitions into the organic phase. (SAM remains in the aqueous phase.)

Other variations on the assay include detection of the non-acylated ACP (i.e., ACP with a free thiol group). Non-acylated ACP can be detected through the use of a thiol reagent such as dithionitrobenzoic acid (DTNB), which releases a highly colored thiolate ($\varepsilon_{412}$ = 13 600 $cm^{-1}$ $M^{-1}$) upon reaction with thiol groups (Ellman, G. L. (1959) *Arch. Biochem. Biophys.* 82:70-77). Another variation of this assay uses an even more sensitive reagent, 4,4'-dithiobipyridyl which has a $\varepsilon_{324}$ = 20 000 $cm^{-1}$ $M^{-1}$ (Jamin, M. *et al.* (1991) *Biochem J.* 280(Pt 2):499-506). Use of DTNB eliminates the need for radioactivity and allows for a continuous assay.

Another variation on the assay includes using a substitute for the substrate C4-ACP. It has already been found that RhlI turns over butanoyl-CoA in lieu of C4-ACP (Parsek, M. R. *et al.* (1999) *Proc. Natl. Acad. Sci. USA.* 96:4360-4365). The $K_M$ for the CoA substrate is 230 μM, compared to 6 μM for C4-ACP, but $v_{max}$ is only one order of magnitude slower. N-Acetylcysteamine represents a truncated moiety of CoA and acylated N-acetylcysteamines often function as substrate analogs for CoA-dependent enzymes (Bayer *et al.* (1995) *Arch Microbiol.* 163(4):310-2; Singh, N. *et al.* (1985) *Biochem Biophys Res Commun.* 131(2):786-92; Whitty, A. (1995) *Biochemistry.* 34(37):11678-89). It will be determined whether butanoyl-N-acetylcysteamine is turned over by RhlI. If so, an assay will be developed for the release of free thiol groups with a thiol reagent such as DTNB. Butanoyl-N-acetylcysteamine is readily synthesized from the commercially available precursors butyrylchloride and N-acetylcysteamine.



butanoyl-N-acetylcysteamine

**LasI activity assay**. In analogy with RhlI, TraI, and LuxI, proposed substrates for LasI are SAM and 3-oxo-C12-ACP. In this assay, compounds are tested for inhibiting the activity of LasI. This assay is based on observations that bacterial strains incubated with $^{14}C$-labeled methionine produce radiolabeled acylated-HSLs, which can be isolated from the culture supernatant and identified by their retention times (in comparison to known standards) when eluted over a high pressure liquid chromatography (HPLC) reversed phase column. A synthase-inhibitor assay has been set up using this methodology.

A *Pseudomonas* strain that expresses *lasI* but not *rhlI*, such as PDO100, is grown in the presence and absence of the test compound (Brint, J. M. *et al.* (1995) *J Bacteriol.* 177(24):7155-63). Cells are pulsed for 10-30 minutes with $^{14}C$-labeled methionine (available from American Radiochemicals) and pelleted by centrifugation. The supernatant liquid is extracted with ethyl acetate and the products separated by HPLC. If the test compound inhibits LasI synthase, the amount of 3-oxo-C12-HSL produced will be significantly reduced when compared to the control.

An *in vitro* assay for LasI activity similar to the radiometric assay used to study RhlI will be developed. The substrates for this assay are $^{14}C$-labeled SAM (available Amersham Pharmacia) and 3-oxo-C12-ACP (similar methodology in Moré, M. I. *et al.* (1996) *Science.* 272(5268):1655-8). LasI activity is monitored by the appearance of radiolabeled 3-oxo-C12-HSL, after extraction into ethyl acetate and scintillation counting. Initially, crude extracts of LasI overexpressed in *E. coli* serve as the source of enzyme. Once a satisfactory assay is in place, a purification protocol will be developed to obtain LasI in a soluble and active form. The purification may involve expression at low levels (low plasmid copy number, weak promoter, low growth temperature) in a *P. aeruginosa rhlI* mutant. Purification will follow standard techniques such as ammonium sulfate precipitation, anion exchange chromatography, cation exchange chromatography and size-exclusion chromatography.

## EXAMPLE 5 IN VIVO ASSAYS TO DETERMINE INHIBITION OF SIGNAL BINDING

*In vivo* assays were also used to determine whether a test compound inhibits signal reception by LasR.

One assay used the *P. aeruginosa* strain QSC102 (Table 3), which responds to the presence of exogenous 3-oxo-C12-HSL by inducing β-galactosidase activity up to 400-fold (Example 1). Cells were grown in the presence of a minimal concentration of 3-oxo-C12-HSL and in the presence and absence of the test compound. If the test compound interferes with signal reception, β-galactosidase activity is reduced. Interference can be a result of any of several mechanisms. The simplest is, if the test compound prevents the 3-oxo-C12-HSL from binding to LasR. Alternatively, the test compound may prevent LasR from binding to DNA or interacting productively with RNA polymerase.

A further *in vivo* assay is used to determine whether a test compound inhibits binding of 3-oxo-C12-HSL to LasR. This assay is based on an observation originally made with LuxR of *Vibrio fischeri*. Namely, the autoinducer binds to *Escherichia coli* cells in which LuxR is produced, provided that LuxR is co-expressed with Hsp60 (Adar *et al.* (1993) *J Biolumin Chemilumin.* 8(5):261-6). This finding was used to develop a competition-assay for binding of inhibitors to LuxR (Schaefer, A. L. *et al.* (1996) *J Bacteriol.* 178(10):2897-901) and LasR (Passador, L. *et al.* (1996) *J Bacteriol.* 178(20):5995-6000). Briefly, cultures of *E. coli* harboring expression plasmids for Hsp60 and LasR (or LuxR) are induced for several hours, at which time an aliquot of cells is added to tritiated signal molecule, alone or in combination with a potential inhibitor. After 10-15 minutes, cells are pelleted by centrifugation, washed, and the amount of radioactivity bound to the cells is determined by scintillation counting.

Plasmids for expression of LasR (pKDT37) (Passador, L. *et al.* (1996) *J Bacteriol.* 178(20):5995-6000) and Hsp60 (pGroESL) have been made. A simple method for preparing $^{14}C$-labeled 3-oxo-C12-HSL has been developed. *E. coli* cells expressing *lasI* excrete $^{14}C$-labeled 3-oxo-C12-HSL into the medium when incubated in the presence of $^{14}C$-labeled methionine. The $^{14}C$-labeled 3-oxo-C12-HSL can be recovered by extraction into ethyl acetate and purified by HPLC. The correct product is

identified by its radioactivity and by the correct HPLC retention time compared to an unlabeled standard.

**EXAMPLE 6 ASSAY FOR INHIBITION OF BIOFILMS**

This assay tests whether compounds useful for inhibiting quorum sensing also inhibit or modulate the formation or growth of biofilms. The LasI/LasR signaling system was found to regulate not only the expression of virulence factors. but also the development of mature biofilms (Davies, D. G. *et al.* (1998) *Science.* 280(5361):295-8). This was demonstrated by using a simple flow-through system, as shown in Figure 12, that allows fresh medium to be pumped through a small chamber in a Plexiglas body.

Cultures of *P. aeruginosa* expressing green fluorescent protein (GFP) were grown in a chamber that was sealed with a coverslip and flushed with fresh medium. Surface attachment and biofilm maturation were determined by examining the coverslip by epifluorescence and confocal microscopy. Both wild type PAO1 and a *rhlI* mutant strain were able to attach to the surface and form the mushroom-shaped structure characteristic of a biofilm. However, a *lasI* mutant that cannot synthesize the signal molecule 3-oxo-C12-HSL was only able to attach to the surface. It did not encase itself in an extracellular matrix or form any kind of three-dimensional structure. It also remained susceptible to 0.2 % sodium dodecyl sulfate, which was used to mimic the susceptibility to a biocide. When the 3-oxo-C12-HSL signal was added back to the *lasI* mutant cells, the wild type phenotype was restored. The cells formed biofilms and remained resistant to sodium dodecyl sulfate.

Accordingly, the bioreactor depicted in Figure 12 is inoculated with wild type *P. aeruginosa* PAO1 that expresses GFP. Test compounds (signaling inhibitors) are added to the flow-through medium to determine whether they prevent formation of the three-dimensional structures typical of a bacterial biofilm. Biofilm formation is monitored using a confocal microscope.

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**Incorporation by Reference**

The contents of all references, patents and published patent applications cited throughout this application, as well as the figures and the sequence listing, are incorporated herein by reference.

**Equivalents**

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments and methods described herein. Such equivalents are intended to be encompassed by the scope of the following claims.

**What is claimed is:**

1. A method for identifying a modulator of quorum sensing signaling in bacteria, said method comprising:

providing a cell which comprises a quorum sensing controlled gene, wherein said cell is responsive to a quorum sensing signal molecule such that a detectable signal is generated;

contacting said cell with a quorum sensing signal molecule in the presence and absence of a test compound;

and detecting a change in the detectable signal to thereby identify said test compound as a modulator of quorum sensing signaling in bacteria.

2. The method of claim 1, wherein said cell further comprises means for generating said detectable signal.

3. The method of claim 2, wherein said signal generation means comprises a reporter gene, and wherein said quorum sensing signal molecule causes transcription of said reporter gene, said transcription providing said detectable signal.

4. The method of claim 3, wherein said reporter gene is operatively linked to a regulatory sequence of said quorum sensing controlled gene.

5. The method of claim 4, wherein said reporter gene is selected from the group consisting of *ADE1, ADE2, ADE3, ADE4, ADE5, ADE7, ADE8, ASP3, ARG1, ARG3, ARG4, ARG5, ARG6, ARG8, ARO2, ARO7, BAR1, CAT, CHO1, CYS3, GAL1, GAL7, GAL10, GFP, HIS1, HIS3, HIS4, HIS5, HOM3, HOM6, ILV1, ILV2, ILV5, INO1, INO2, INO4, lacZ, LEU1, LEU2, LEU4, luciferase, LYS2, MAL, MEL, MET2, MET3, MET4, MET8, MET9, MET14, MET16, MET19, OLE1, PHO5, PRO1, PRO3, THR1, THR4, TRP1, TRP2, TRP3, TRP4, TRP5, URA1, URA2, URA3, URA4, URA5* and *URA10*.

6. The method of claim 5, wherein said reporter gene is *lacZ* or *GFP*.

7. The method of claim 1, wherein said cell does not express said quorum sensing signal molecule.

8. The method of claim 7, wherein said quorum sensing signal molecule is produced by a second cell.

9. The method of claim 1, wherein said cell is a prokaryote or eukaryote.

10. The method of claim 9, wherein said cell is a bacterium.

11. The method of claim 8, wherein said second cell is a prokaryote or eukaryote.

12. The method of claim 11, wherein said second cell is a bacterium.

13. The method of claims 10 or 12, wherein said bacterium is a gram negative bacterium.

14. The method of claim 13, wherein said gram negative bacterium is *Pseudomonas aeruginosa*.

15. The method of claim 10, wherein said bacterium is a mutant strain of *Pseudomonas aeruginosa* which comprises a regulatory sequence of a quorum sensing controlled gene operatively linked to a reporter gene, wherein in said mutant strain, *lasI* and *rhlI* are inactivated.

16. The method of claim 12, wherein said second cell is wild type *Pseudomonas aeruginosa*.

17. The method of claim 1, wherein said quorum sensing controlled gene is endogenous to said cell.

18. The method of claim10, wherein said quorum sensing controlled gene encodes a virulence factor.

19. The method of claim 10, wherein said quorum sensing controlled gene encodes a polypeptide which inhibits a bacterial host defense mechanism.

20. The method of claim 10, wherein said quorum sensing controlled gene encodes a polypeptide which regulates biofilm formation.

21. The method of claim 1, wherein said quorum sensing signal molecule is an autoinducer of said quorum sensing controlled gene.

22. The method of claim 21, wherein said autoinducer is a homoserine lactone.

23. The method of claim 22, wherein said test compound is a homoserine lactone analog.

24. The method of claim 1, wherein said modulator inhibits an enzyme involved in the synthesis by said bacterium of said quorum sensing signal molecule.

25. The method of claim 1, wherein said modulator inhibits reception of said quorum sensing signal molecule by said bacterium.

26. The method of claim 1, wherein said modulator scavenges said quorum sensing signal molecule.

27. A method for identifying a modulator of quorum sensing signaling in *Pseudomonas aeruginosa*, said method comprising:

providing a wild type strain of *Pseudomonas aeruginosa* which produces a quorum sensing signal molecule;

providing a mutant strain of *Pseudomonas aeruginosa* which comprises a reporter gene operatively linked to a regulatory sequence of a quorum sensing controlled gene, wherein said mutant strain is responsive to said quorum sensing signal molecule produced by said wild type strain, such that a detectable signal is generated;

contacting said mutant strain with said quorum sensing signal molecule and a test compound; and

detecting a change in the detectable signal to thereby identify said test compound as a modulator of quorum sensing signaling in *Pseudomonas aeruginosa.*

28. The method of claim 27, wherein in said mutant strain, *lasI* and *rhlI* are inactivated.

29. The method of claim 27, wherein said reporter gene is *lacZ* or *GFP*.

30. The method of claim 29, wherein said reporter gene is *lacZ*.

31. The method of claim 29, wherein said reporter gene is *GFP*.

32. The method of claim 31, wherein said reporter gene is a variant of *GFP*.

33. The method of claim 32, wherein said variant is GFPmut2.

34. The method of claim 27, wherein said mutant strain of *Pseudomonas aeruginosa* comprises a promoterless reporter gene inserted at a genetic locus in the chromosome of said *Pseudomonas aeruginosa*, wherein said locus comprises a nucleotide sequence selected from the group consisting of: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35 and SEQ ID NO:36.

35. The method of claim 34, wherein said promoterless reporter gene is inserted in said chromosome at a locus comprising a nucleotide sequence selected from the group consisting of: SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:28 and SEQ ID NO:35.

36. The method of claim 34, wherein said reporter gene is contained in a transposable element.

37. A mutant strain of *Pseudomonas aeruginosa* comprising a promoterless reporter gene inserted at a genetic locus in the chromosome of said *Pseudomonas aeruginosa*, wherein said locus comprises a nucleotide sequence selected from the group consisting of: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35 and SEQ ID NO:36.

38. The mutant strain of claim 37, wherein said reporter gene is contained in a transposable element.

39. The mutant strain of claim 37, wherein *lasI* and *rhlI* are inactivated.

40. The mutant strain of claim 37, wherein said strain is responsive to a quorum sensing signal molecule such that a detectable signal is generated by said reporter gene.

41. The mutant strain of claim 37, wherein said reporter gene is *lacZ* or *GFP*.

42. The method of claim 41, wherein said reporter gene is a variant of *GFP*.

43. The method of claim 42, wherein said variant is GFPmut2.

44. A method for identifying a modulator of a quorum sensing signaling in *Pseudomonas aeruginosa*, said method comprising:

providing a wild type strain of *Pseudomonas aeruginosa* which produces a quorum sensing signal molecule;

providing a mutant strain of *Pseudomonas aeruginosa* which comprises a promoterless reporter gene inserted at a genetic locus in the chromosome of said *Pseudomonas aeruginosa*, wherein said locus comprises a nucleotide sequence selected from the group consisting of: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35 and SEQ ID NO:36; and wherein said mutant strain is responsive to said quorum

sensing signal molecule produced by said wild type strain, such that a detectable signal is generated by said reporter gene;

contacting said mutant strain with said quorum sensing signal molecule and a test compound; and

detecting a change in the detectable signal to thereby identify said test compound as a modulator of quorum sensing signaling in *Pseudomonas aeruginosa*.

45. The method of claim 44, wherein said reporter gene is contained in a transposable element.

46. An isolated nucleic acid molecule comprising a nucleotide sequence, said nucleotide sequence comprising:

a regulatory sequence derived from the genome of *Pseudomonas aeruginosa*, wherein said regulatory sequence regulates a quorum sensing controlled genetic locus of the *Pseudomonas aeruginosa* chromosome, and wherein said locus comprises a nucleotide sequence selected from the group consisting of: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35 and SEQ ID NO:36; and

a reporter gene operatively linked to said regulatory sequence.

47. An isolated nucleic acid molecule comprising a quorum sensing controlled genetic locus derived from the genome of *Pseudomonas aeruginosa* , wherein said locus comprises a nucleotide sequence selected from the group consisting of: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35 and SEQ ID NO:36, operatively linked to a reporter gene.

48. An isolated nucleic acid molecule comprising a polynucleotide having at least 80% identity to a quorum sensing controlled genetic locus derived from the genome of *Pseudomonas aeruginosa*, wherein said locus comprises a nucleotide sequence selected from the group consisting of: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35 and SEQ ID NO:36, operatively linked to a reporter gene.

49. An isolated nucleic acid molecule comprising a polynucleotide that hybridizes under stringent conditions to the complement of a nucleotide sequence comprising a quorum sensing controlled genetic locus derived from the genome of *Pseudomonas aeruginosa* , wherein said locus comprises a nucleotide sequence selected from the group consisting of: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35 and SEQ ID NO:36, operatively linked to a reporter gene.

50. The nucleic acid molecule of any one of claims 46, 47, 48 and 49, wherein said reporter gene is contained in a transposable element.

51. A vector comprising the isolated nucleic acid molecule of any one of claims 46, 47, 48 and 49.

52. A cell containing an isolated nucleic acid molecule of any one of claims 46, 47, 48 and 49.

53. A method for identifying a modulator of quorum sensing signaling in bacteria, said method comprising:

providing the cell of claim 52, wherein said cell is responsive to a quorum sensing signal molecule such that a detectable signal is generated;

contacting said cell with a quorum sensing signal molecule in the presence and absence of a test compound;

and detecting a change in the detectable signal to thereby identify said test compound as a modulator of quorum sensing signaling in bacteria.

54. A compound which inhibits quorum sensing signaling in *Pseudomonas aeruginosa*, said compound having been identified by the method of claim 28.

55. The compound of claim 54, which inhibits quorum sensing signaling in *Pseudomonas aeruginosa* by inhibiting an enzyme involved in the synthesis of a quorum sensing signal molecule, by interfering with quorum sensing signal reception , or by scavenging the quorum sensing signal molecule.

56. A method for identifying a quorum sensing controlled gene in bacteria, said method comprising:

providing a cell which is responsive to a quorum sensing signal molecule such that expression of a quorum sensing controlled gene is modulated, and wherein modulation of the expression of said quorum sensing controlled gene generates a detectable signal;

contacting said cell with a quorum sensing signal molecule;

and detecting a change in the detectable signal to thereby identify a quorum sensing signaling controlled gene in bacteria.

57. The method of claim 56, wherein said cell further comprises means for generating said detectable signal.

58. The method of claim 57, wherein said signal generation means comprises a reporter gene, and wherein modulation of the expression of said quorum sensing controlled gene modulates transcription of said reporter gene, said transcription providing said detectable signal.

59. The method of claim 58, wherein said reporter gene is operatively linked to a regulatory sequence of said quorum sensing controlled gene.

60. The method of claim 58, wherein said reporter gene is opertively linked to said quorum sensing controlled gene.

61. The method of either of claims 59 and 60, wherein said reporter gene is contained in a transposable element.

62. The method of claim 58, wherein said reporter gene is selected from the group consisting of *ADE1, ADE2, ADE3, ADE4, ADE5, ADE7, ADE8, ASP3, ARG1, ARG3, ARG4, ARG5, ARG6, ARG8, ARO2, ARO7, BAR1, CAT, CHO1, CYS3, GAL1, GAL7, GAL10, GFP, HIS1, HIS3, HIS4, HIS5, HOM3, HOM6, ILV1, ILV2, ILV5, INO1, INO2, INO4, lacZ, LEU1, LEU2, LEU4, luciferase, LYS2, MAL, MEL, MET2, MET3, MET4, MET8, MET9, MET14, MET16, MET19, OLE1, PHO5, PRO1, PRO3, THR1, THR4, TRP1, TRP2, TRP3, TRP4, TRP5, URA1, URA2, URA3, URA4, URA5* and *URA10*.

63. The method of claim 56, wherein said quorum sensing signal molecule is produced by a second cell.

64. The method of claim 63, wherein said second cell is a prokaryote or eukaryote.

65. The method of claim 64, wherein said second cell is a bacterium.

66. The method of claim 56, wherein said cell is a prokaryote or eukaryote.

67. The method of claim 66, wherein said cell is a bacterium.

68. The method of either of claims 65 and 67, wherein said bacterium is a gram negative bacterium.

69. The method of claim 68, wherein said gram negative bacterium is *Pseudomonas aeruginosa*.

70. The method of claim 67, wherein said bacterium is a mutant strain of *Pseudomonas aeruginosa* in which *lasI* and *rhlI* are inactivated.

71. The method of claim 65, wherein said second cell is wild type *Pseudomonas aeruginosa*.

72. The method of claim 56, wherein said quorum sensing signal molecule is an autoinducer of said quorum sensing controlled gene.

73. The method of claim 72, wherein said autoinducer is a homoserine lactone, or an analog thereof.

74. The method of claim 56, wherein said quorum sensing signal molecule induces the expression of said quorum sensing controlled gene.

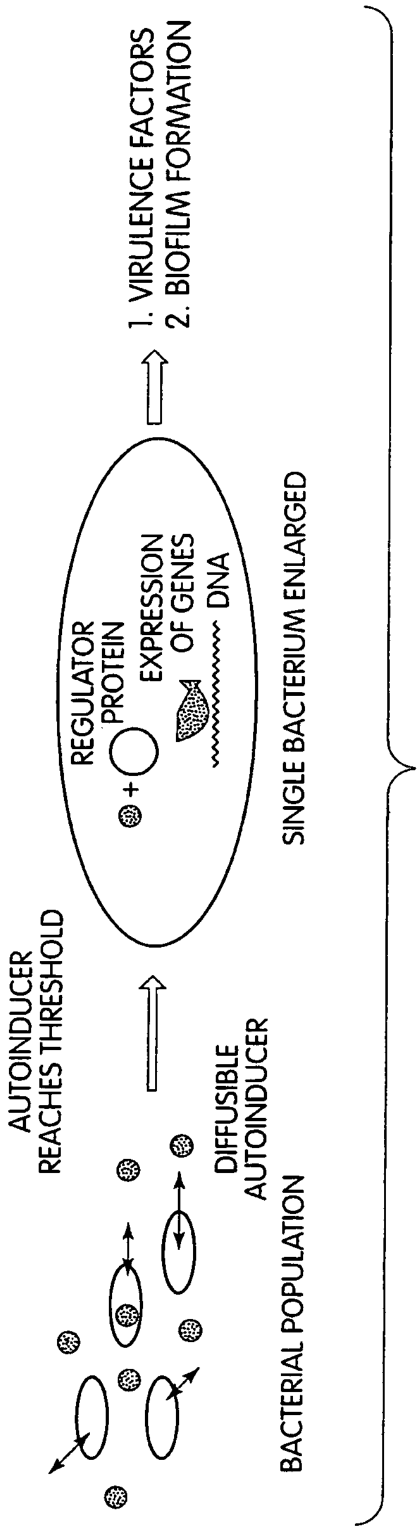
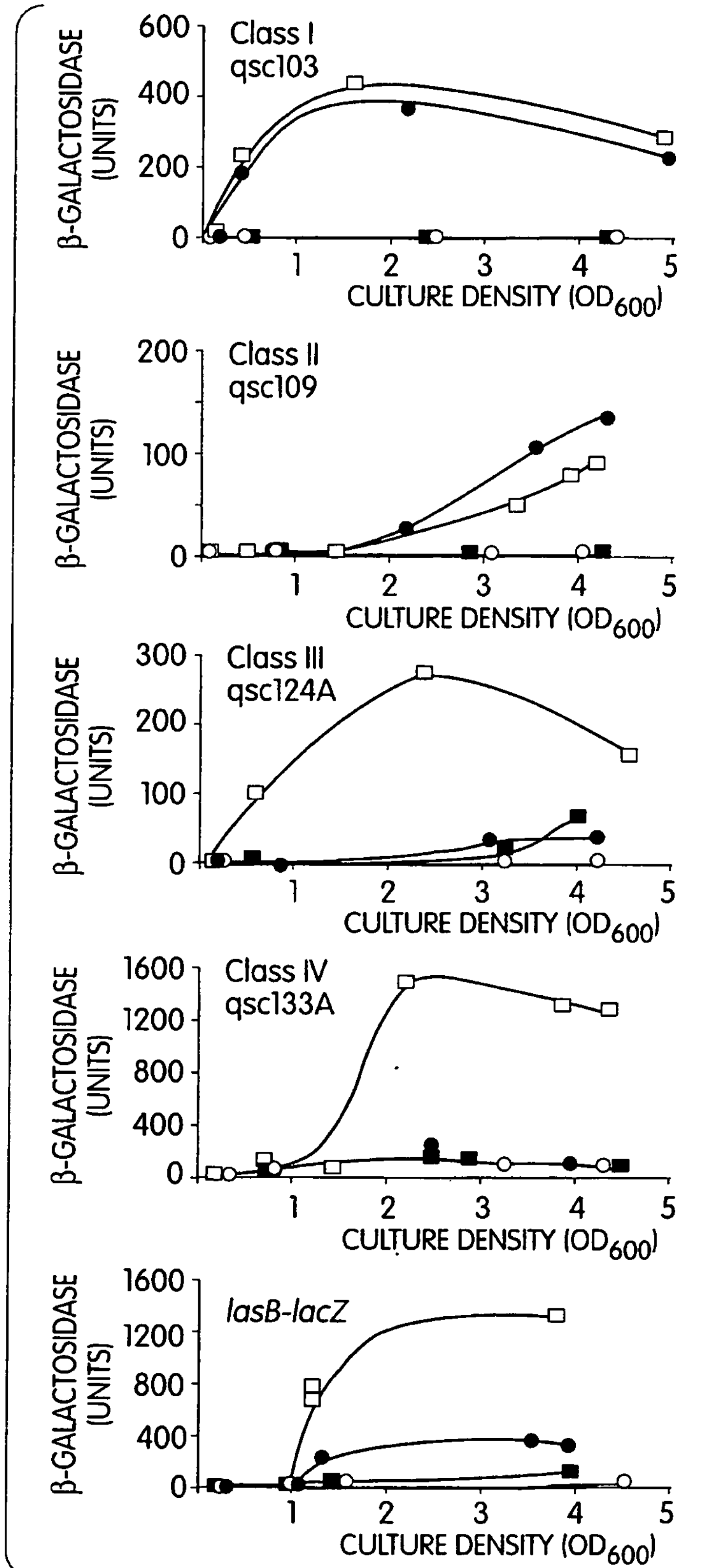

BACTERIAL POPULATION
DIFFUSIBLE AUTOINDUCER
AUTOINDUCER REACHES THRESHOLD
REGULATOR PROTEIN
EXPRESSION OF GENES
DNA
SINGLE BACTERIUM ENLARGED
1. VIRULENCE FACTORS
2. BIOFILM FORMATION

Fig. 1



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2
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5
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100
Class III
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300
Class IV
qsc133A
1600
1200
800
lasB-lacZ


Fig. 2

3/13

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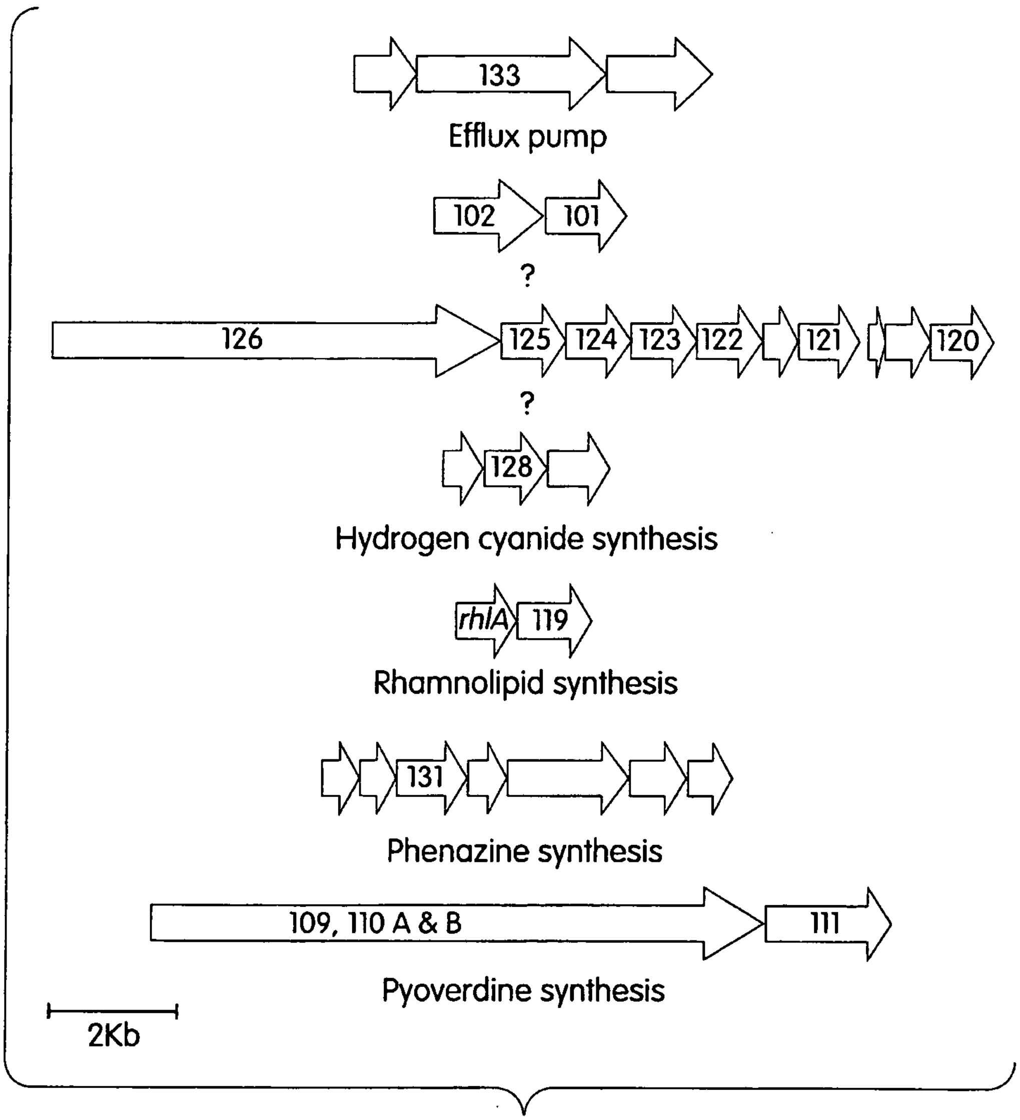
Fig. 3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101
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125
124
123
122
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120
?
128
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Rhamnolipid synthesis
131
Phenazine synthesis
109, 110 A & B
111
Pyoverdine synthesis
2Kb

Fig. 4

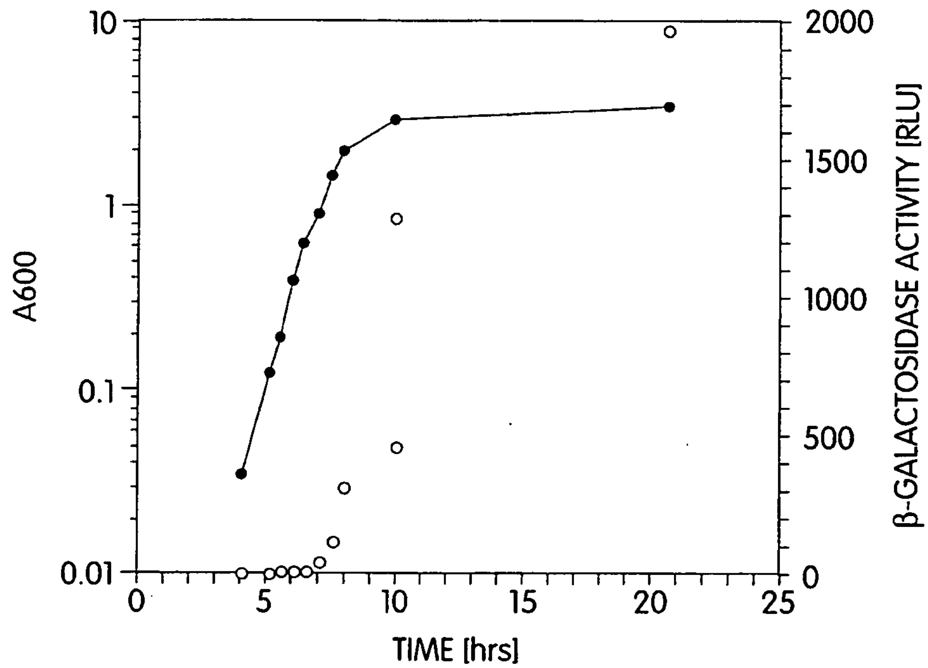
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Fig. 5

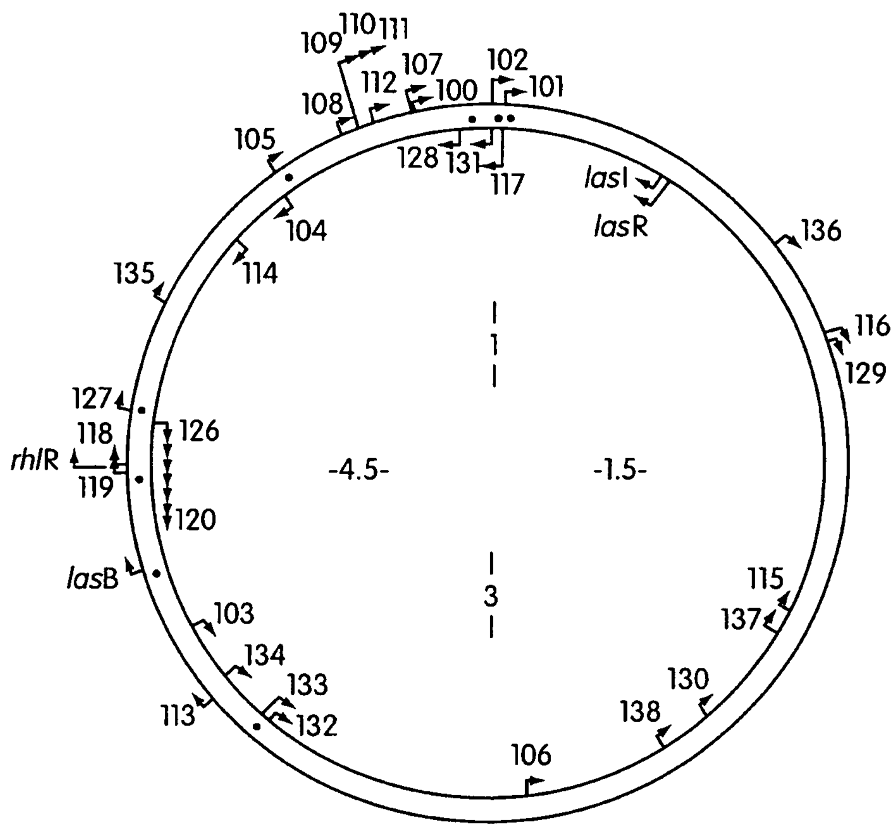

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Fig. 6

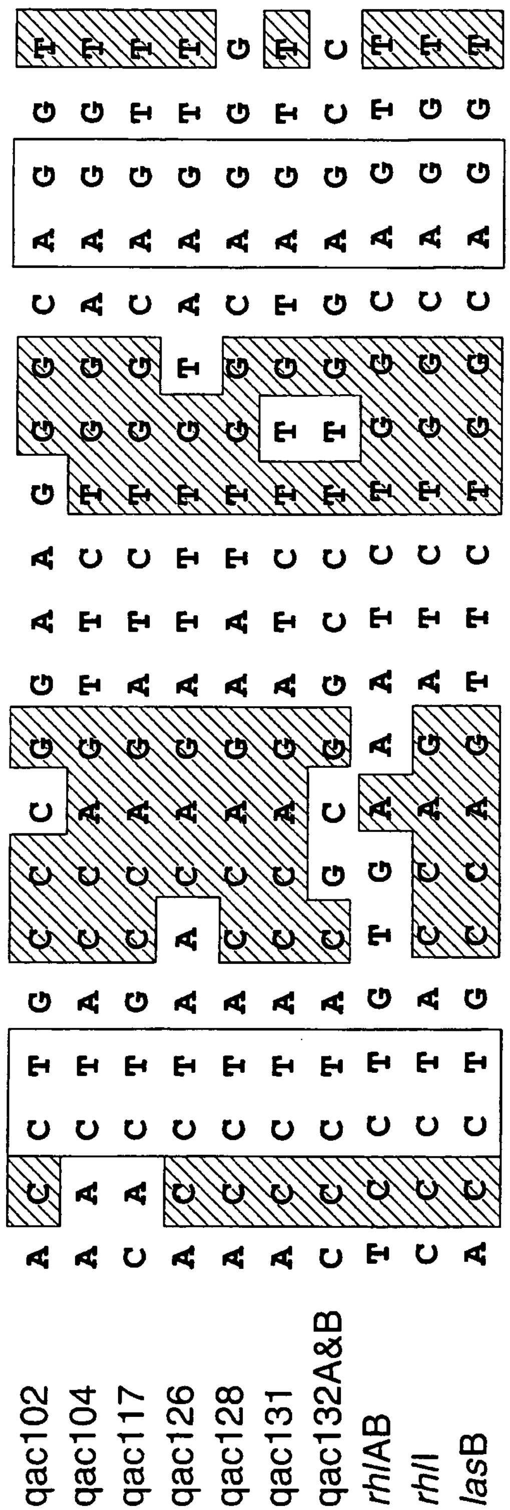
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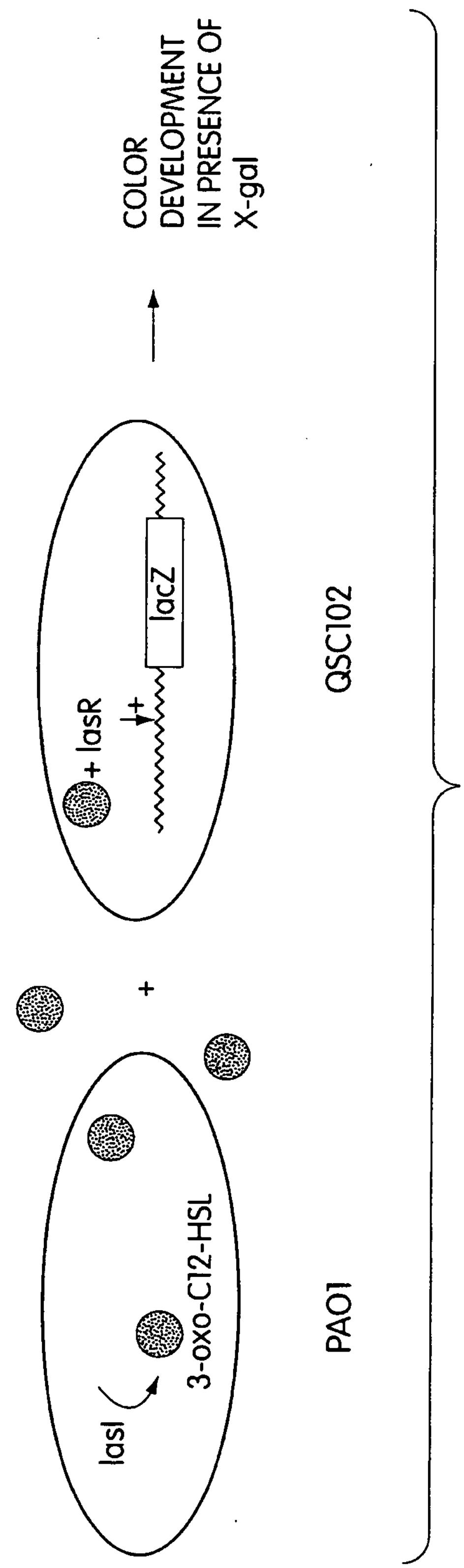
Fig. 7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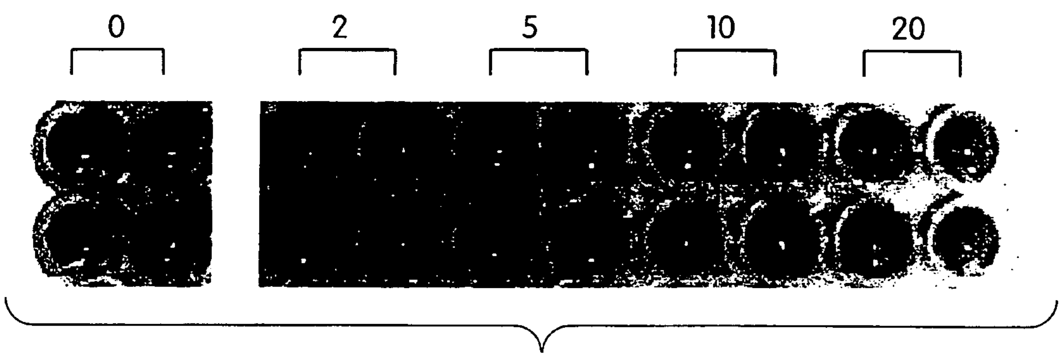
COLOR
DEVELOPMENT
IN PRESENCE OF
X-gal

Fig. 8

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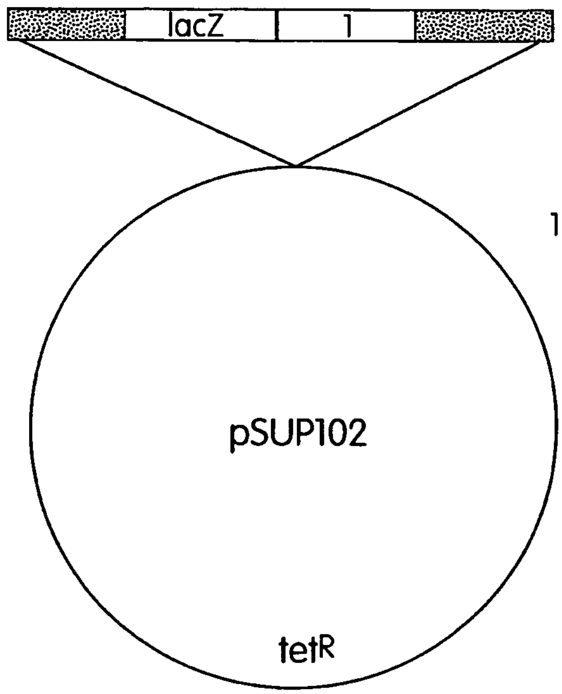
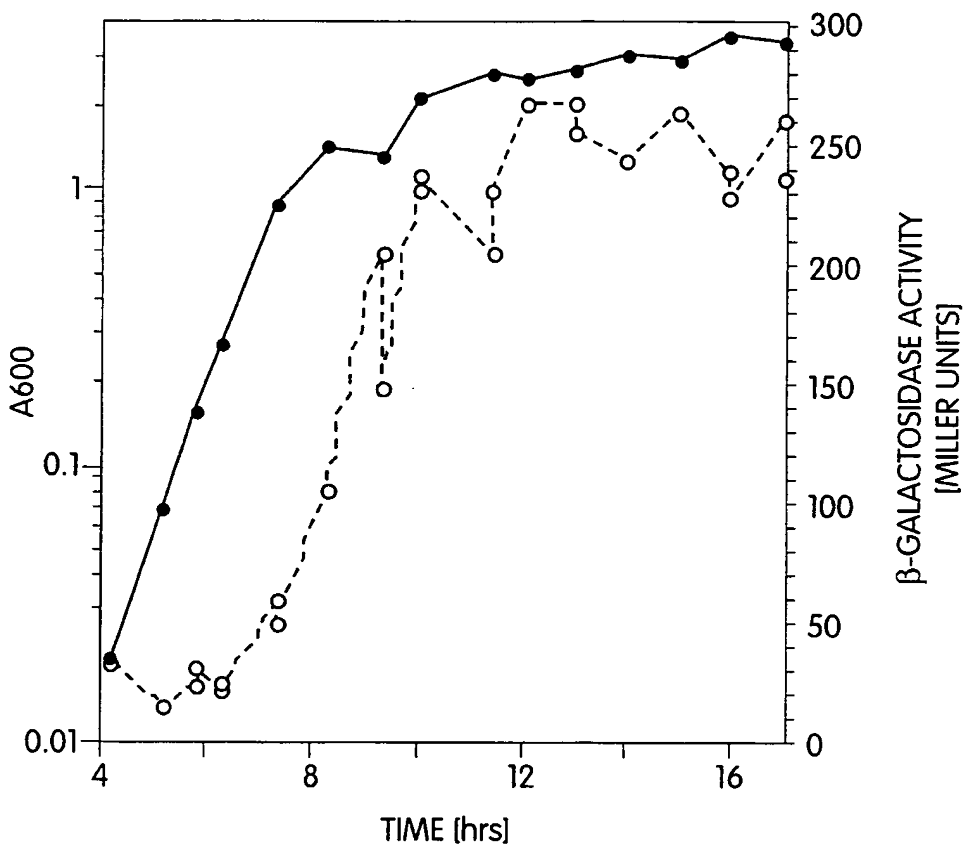



1: GENTAMICIN RESISTANCE

Fig. 10A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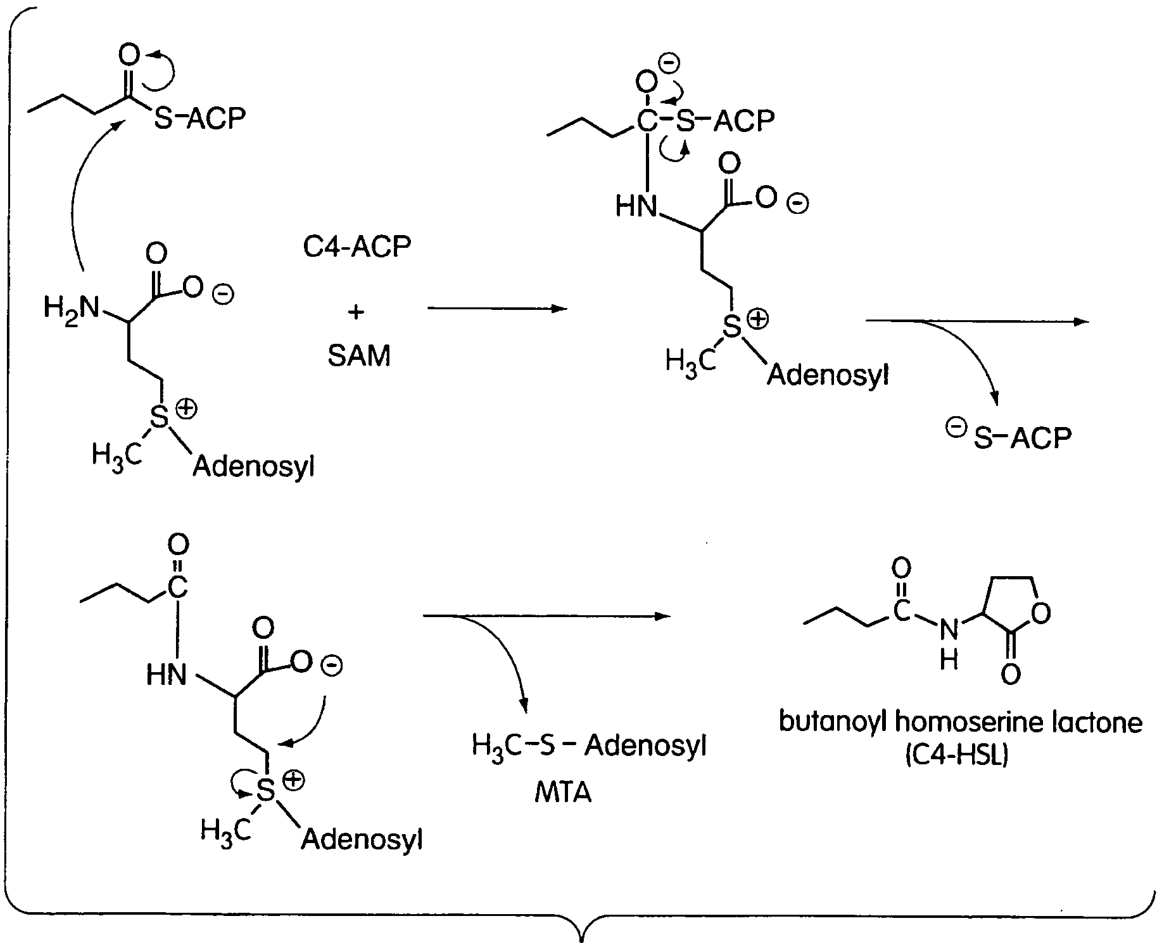
β-GALACTOSIDASE ACTIVITY
[MILLER UNITS]

Fig. 10B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(C4-HSL)

Fig. 11

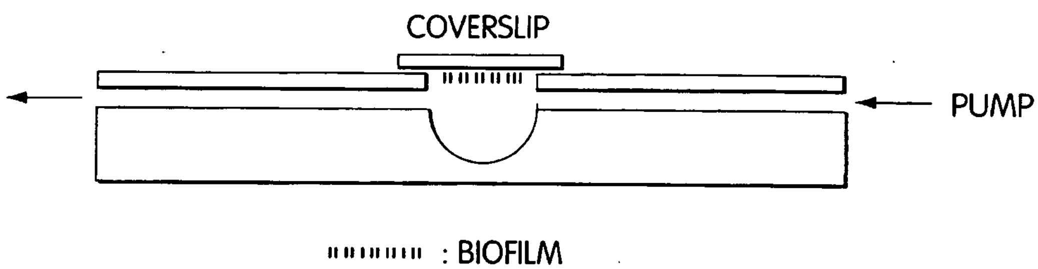


Fig. 12

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<110> University of Iowa Research Foundation et al.

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(10) International Publication Number
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(21) International Application Number: PCT/US00/24141

(22) International Filing Date: 1 September 2000 (01.09.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
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US 60/153,022 (CON)
Filed on 3 September 1999 (03.09.1999)

(71) Applicants *(for all designated States except US)*: THE UNIVERSITY OF IOWA RESEARCH FOUNDATION [US/US]; 214 Technology Innovation Center, Iowa City, IA 52242 (US). QUORUM SCIENCES, INC. [US/US]; 11010 Torreyana Road, San Diego, CA 92121 (US).

(72) Inventors; and
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(74) Agents: **HANLEY, Elizabeth, A.** et al.; Lahive & Cockfield, LLP, 28 State Street, Boston, MA 02109 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

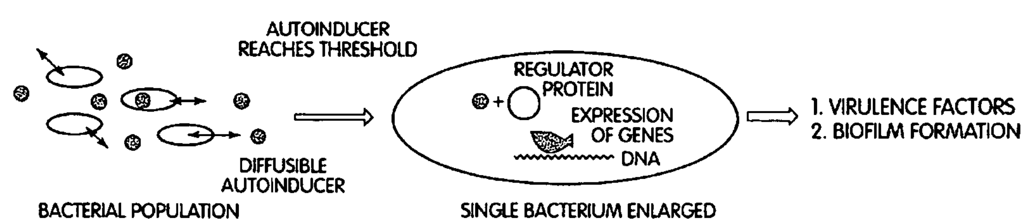
(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

**Published:**
— *with international search report*

(88) Date of publication of the international search report: 15 August 2002

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

(54) Title: QUORUM SENSING SIGNALING IN BACTERIA




(57) Abstract: The invention provides methods for identifying a modulator of quorum sensing signaling in bacteria, and for identifying a quorum sensing controlled gene in bacteria. In addition, the invention provides quorum sensing controlled genetic loci in *(Pseudomonas aeruginosa)*. Novel indicator strains and vectors for engineering the strains for use in the method of the invention are also provided.

WO 01/018248 A3

| INTERNATIONAL SEARCH REPORT | International Application No PCT/US 00/24141 |
|---|---|

**A. CLASSIFICATION OF SUBJECT MATTER**
IPC 7 C12Q1/68 C07K14/21 C12N15/78

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, SEQUENCE SEARCH, BIOSIS, EMBL

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X<br><br>Y | US 5 591 872 A (PEARSON JAMES P ET AL)<br>7 January 1997 (1997-01-07)<br>the whole document<br>--- | 1-14,<br>16-26<br>15,27-33 |
| Y | DE KIEVIT TERESA R ET AL: "Quorum sensing, gene expression, and Pseudomonas biofilms."<br>METHODS IN ENZYMOLOGY,<br>vol. 310, September 1999 (1999-09), pages 117-131, XP001052861<br>1999 Academic Press Inc.; Academic Press Ltd. 525 B Street, Suite 1900, San Diego, CA, 92101-4495, USA; 24-28 Oval Road, London, NW1 7DX, UK<br>ISBN: 0-12-182211-7<br>the whole document<br>--- | 15,27-33 |

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 25 January 2002 | 13. 05. 2002 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | REUTER, U |

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | PEARSON JAMES P ET AL: "Roles of Pseudomonas aeruginosa las and rhl quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes." JOURNAL OF BACTERIOLOGY, vol. 179, no. 18, 1997, pages 5756-5767, XP002188055 ISSN: 0021-9193 page 5757, right-hand column -page 5759, left-hand column; table 1 page 5762, right-hand column -page 5764, left-hand column --- | 15,27-33 |
| Y | DE KIEVIT TERESA ET AL: "RsaL, a novel repressor of virulence gene expression in Pseudomonas aeruginosa." JOURNAL OF BACTERIOLOGY, vol. 181, no. 7, April 1999 (1999-04), pages 2175-2184, XP002188056 ISSN: 0021-9193 the whole document --- | 27-33 |
| X | PIERSON LELAND S III ET AL: "Phenazine antibiotic biosynthesis in Pseudomonas aureofaciens 30-84 is regulated by PhzR in response to cell density." JOURNAL OF BACTERIOLOGY, vol. 176, no. 13, 1994, pages 3966-3974, XP001053143 ISSN: 0021-9193 the whole document --- | 48,49, 51,52 |
| A | WO 98 57618 A (UNIV MONTANA RES DEV INST ;DAVIES DAVID G (US); COSTERTON JOHN WIL) 23 December 1998 (1998-12-23) the whole document --- | 1-53 |
| A | LATIFI A ET AL: "MULTIPLE HOMOLOGUES OF LUXR AND LUXL CONTROL EXPRESSION OF VIRULENCE DETERMINANTS AND SECONDARY METABOLITES THROUGH QUORUM SENSING IN PSEUDOMONAS AERUGINOSA PAO1" MOLECULAR MICROBIOLOGY, BLACKWELL SCIENTIFIC, OXFORD, GB, vol. 17, no. 2, 1995, pages 333-343, XP000857602 ISSN: 0950-382X the whole document --- | 27-53 |

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | DATABASE EMBL [Online] EMBL; Database entry PAAF5404 acc nb AF005404, 4 July 1997 (1997-07-04) MAVRODI D. ET AL.: "Pseudomonas aeruginosa pyocyanine biosynthesis operon" XP002188061 sequence comprises Seq.ID.No 1 abstract --- | 27-53 |
| A | CHAPON-HERVE VIRGINIE ET AL: "Regulation of the xcp secretion pathway by multiple quorum-sensing modulons in Pseudomonas aeruginosa." MOLECULAR MICROBIOLOGY, vol. 24, no. 6, 1997, pages 1169-1178, XP001052865 ISSN: 0950-382X the whole document --- | 1-53 |
| A | PESCI EVERETT C ET AL: "Regulation of las and rhl Quorum sensing in Pseudomonas aeruginosa." JOURNAL OF BACTERIOLOGY, vol. 179, no. 10, 1997, pages 3127-3132, XP002188058 ISSN: 0021-9193 the whole document ----- | 1-53 |

## INTERNATIONAL SEARCH REPORT

ıational application No.
PCT/US 00/24141

**Box I Observations where certain claims were found unsearchabl (C ntinuation of item 1 of first sheet)**

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [ ] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. [X] Claims Nos.: 54,55
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [X] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-26 (complete); 27-53 (partially)

**Remark on Protest**

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 54,55

Present claims 54 and 55 relate to a compound defined by reference to a desirable characteristic or property, namely being identifyable by a certain method.
The claims cover all compounds having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such compounds. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the compound by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, no search has been carried out for said claims.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-26 (all completely), 27-53 (all partially)

   Invention 1:

   A method for identifying a modulator of quorum sensing signalling in bacteria, an isolated nucleic acid molecule being defined by Seq Id NO 1 operatively linked to a reporter gene, a vector comprising said nucleic acid, a cell containing said nucleic acid, a mutant strain of Pseudomonas aeruginosa comprising a promoterless reporter gene inserted in the genetic locus comprising said sequence, and a method for identifying a modulator of quorum sensing signaling in Pseudomonas aeruginosa using said mutant.

2. Claims: 27-53 (all partially)

   Inventions 2-36:

   An isolated nucleic acid molecule being defined by Seq Id NO n, wherin n ranges from 2-36, operatively linked to a reporter gene, a vector comprising said nucleic acid, a cell containing said nucleic acid, a mutant strain of Pseudomonas aeruginosa comprising a promoterless reporter gene inserted in the genetic locus comprising said sequence, and a method for identifying a modulator of quorum sensing signaling in Pseudomonas aeruginosa using said mutant.

3. Claims: 56-74 (all completely)

   Invention 37:

   A method for identifying a quorum sensing controlled gene in bacteria

# INTERNATIONAL SEARCH REPORT

Ir  onal Application No
PCT/US 00/24141

| Patent document cited in search report | | Publication date | Patent family member(s) | | Publication date |
|---|---|---|---|---|---|
| US 5591872 | A | 07-01-1997 | US | 6057288 A | 02-05-2000 |
| WO 9857618 | A | 23-12-1998 | AU | 7977698 A | 04-01-1999 |
| | | | AU | 8258998 A | 04-01-1999 |
| | | | EP | 0994961 A2 | 26-04-2000 |
| | | | WO | 9857618 A1 | 23-12-1998 |
| | | | WO | 9858075 A2 | 23-12-1998 |